

**TEMPORAL EFFECTS OF FESCUE TOXICOSIS AND HEAT
STRESS ON RAT PHYSIOLOGY AND HEPATIC GENE
EXPRESSION**

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LIST OF ABBREVIATIONS

5-HT	5- Hydroxytryptamine
Ac	Activity count
ADFI	Average daily feed intake
ADG	Average daily gain
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
BASE	Bioarray soft ware environment
BUN	Blood urea nitrogen
BW	Body weight
CAT	Catalase
C _t	Cycle number in RT-PCR at which the fluorescence passes the threshold
CYP	Cytochrome P450 isoform
DMEM	Dulbecco's modified Eagle's medium
D2	Dopamine-2 receptor
E-	Endophyte-free fescue diet
E+	Endophyte-infected fescue diet
E-HS	Rats fed E- diet and maintained under heat stress conditions
E+HS	Rats fed E+ diet and maintained under heat stress conditions
E-TN	Rats fed E- diet and maintained under thermoneutral conditions
E+TN	Rats fed E+ diet and maintained under thermoneutral conditions
EV	Ergovaline
FCE	Feed conversion efficiency
FDR	False discovery rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
FI	Feed intake
GPx	Glutathione peroxidase
GSH	Glutathione (Reduced)
GSSG	Glutathione disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H ₂ O ₂	Hydrogen peroxide
H&E	Hematoxylin and eosin
HS	Heat stress
HSF	Heat shock factor
HSP	Heat shock protein
HO-1	Heme oxygenase-1
IHC	Immunohistochemistry
KRB	Krebs-Ringer-bicarbonate buffer
LH	Long-term-heat stress
LPS	Lipopolysacharide
LTHA	Long-term heat acclimation
MDMA	3, 4-methylenedioxymethamphetamine
MDR 1	Multidrug resistance gene 1

MRP 2	Multidrug resistance associated protein 2
NO	Nitric oxide
PAS	Periodic Acid-Schiff
PCNA	Proliferating cell nuclear antigen
PRL	Prolactin
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RT-PCR	Real-time PCR
RXR	Retinoid X receptor
SAM	Significance Analysis of Microarray
SDS	Sodium dodecyl sulfate
SH	Short-term-heat stress
SOD	Superoxide dismutase
SSC	Standard saline citrate
STHA	Short-term heat acclimation
SUMO-1	Small ubiquitin-like modifier 1
T3 and T4	Triiodothyronine and Thyroxine
Ta	Ambient temperature
TBARS	Thiobarbituric acid reactive substrates
Tc	Core body temperature
THI	Temperature-humidity index
TN	Thermoneutrality
TRH	Thyroid stimulating hormone-releasing hormone
TUNEL	Terminal dUTP nick-end labeling

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Dr. Don Spiers, Dissertation Supervisor

ABSTRACT

Ingestion of endophyte (*Neotyphodium coenophialum*) - infected tall fescue diet (E+) often impairs animal health and productivity. These effects or problems are collectively termed fescue toxicosis. Clinical signs associated with fescue toxicosis are mainly dependent on the ambient temperature and period of exposure. Characteristics of fescue toxicosis include reduced feed intake (FI), growth, and serum prolactin level. Thermoregulatory shifts are hypothermia under cold or thermoneutral (TN; 21°C) conditions and hyperthermia during heat stress (HS; 31°C). Rats exhibit similar characteristics of fescue toxicosis, and are very sensitive responders to E+ treatment under both TN and HS conditions. The primary objective of the present studies was to determine the temporal (short vs long-term) physiological changes associated with the intake of E+ or uninfected fescue seed diet (E-) under TN and HS conditions. The secondary objective was to identify the temporal effects of fescue toxicosis on hepatic gene expression in TN and HS environments. The final objective was to determine the effects of fescue toxicosis on hepatic detoxification pathways in these environments.

Three experiments were performed during which different rats were initially fed either E+ or E- fescue seed diets, and maintained at TN for five days (pre-heat stress/TN). In two experiments the exposure was extended under TN or HS conditions for either three days (short-term HS) or three weeks (long-term HS). The schedule in all three experiments consisted initially of an eight-day recovery period after intraperitoneal implantation of telemetric transmitters to measure core temperature (T_c) and activity count (Ac), followed by a five-day pretreatment period (d -5 to -1), during which all rats received E- diet (Spiers et al., 2005). Rats in Experiment 1 were randomly assigned to either E- (n = 12) or E+ treatment (n = 12) groups and administered treatment at TN for 5 d (d 1 to 5; Figure 1) to determine the fescue toxicosis response in the absence of heat stress. During short-term HS (Experiment 2), rats were exposed to heat stress (n = 6; two groups: E- HS and E+ HS) for three days. During long-term HS (Experiment 3), rats were divided at random into four groups at the end of the TN period (n = 6; four groups: E- TN, E- HS, E+ TN, and E+ HS) to determine adaptive responses to heat stress and fescue toxicosis. At the end of each treatment period, rats from each group were euthanized and blood and selected visceral organs (i.e., liver, adrenal, kidney, heart, and testes) were collected from each animal. Wet organ weights were also measured at this time. Intake of E+ diet did not change daily T_c in rats at TN, but increased T_c with short- and long-term HS exposures. In addition, this intake reduced FI, FCE, daily gain, serum prolactin and cholesterol levels during both TN and HS exposures. Under TN conditions, rats adapted to the E+ diet, as reflected by the complete recovery in FI, FCE, and partial recovery in weight gain. However, rats simultaneously exposed to a second stressor (i. e., HS) failed to show a similar rate of recovery, suggesting a greater shift from normalcy

with multiple stressors. Moreover, the long-term depressant effect of HS seemed to have a greater influence on physiological parameters than intake of E+ diet, which seemed to elicit a more transient response.

Microarray analysis was used to identify shifts in hepatic gene expression associated with affected physiological processes as a result of E+ diet intake. Data from the analyses were analyzed using a two-step ANOVA model and the results validated using real-time PCR. In Experiment 1, microarray analysis revealed that E+ treatment down-regulated various genes associated with energy metabolism, growth and development, antioxidant protection, immune function, and chaperone activity. Furthermore, this treatment up-regulated genes coding for gluconeogenesis, detoxification, and biotransformation. Partial adaptation in FI, FCE and growth rate that was observed in E+ rats by the end of Experiment 1 could be partially attributed to the up-regulation of genes associated with biotransformation and detoxification. However, down-regulation of genes coding for antioxidant, immune, and chaperone functions, along with energy metabolism, could contribute to the toxicological signs observed with fescue toxicosis. This study demonstrated that even short-term intake of E+ diet under TN conditions can result in altered hepatic gene expression contributing to the clinical signs associated with fescue toxicosis.

Microarray results obtained from Experiment 1 supported the well proven notion that the liver is one of the major target organs for fescue toxicosis. Effects of E+ intake on hepatic detoxification pathways were evaluated using liver tissue samples from Experiment 1. Hepatic gene expression of various CYPs, selected nuclear receptors associated with the CYP induction, and antioxidant enzymes were measured using real-time PCR. Hepatic

expression of CYP, antioxidant and proliferating cell nuclear antigen (PCNA) proteins were measured using western blots. The CYP3A1 protein expression was evaluated using primary rat hepatocellular cultures treated with ergovaline, one of the major ergot alkaloids produced by fescue endophyte, in order to assess the direct effect of ergot alkaloids on CYP3A1 induction. The enzyme activities of selected antioxidants were assayed spectrophotometrically. While hepatic CYP and nuclear receptor expression were increased in E+ rats, the expression and activity of antioxidant enzymes were reduced. This could potentially lead to increased oxidative stress, which might be responsible for the decrease in hepatocellular proliferation after E+ diet intake. This study demonstrated that even short-term exposure to ergot alkaloids under TN conditions can potentially induce hepatic oxidative stress which can contribute to the pathogenesis of fescue toxicosis.

The effects of fescue toxicosis on hepatic gene expression were evaluated under short-term HS conditions in Experiment 2. This experiment also compared the effects of E+ treatment under TN conditions versus under short-term HS conditions. Furthermore, protein expression of hepatic cytochrome P450 3A4 (CYP3A4) was evaluated. Hepatic enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were also determined. Hepatic apoptosis rates were measured using the TUNEL assay. Results obtained from microarray analysis showed that genes coding for antioxidant system, and carbohydrate and lipid metabolisms were down-regulated, and CYP2c37 was up-regulated in E+ compared to E- under HS. However, only a few genes were differentially expressed for each pathway, and therefore it was difficult to draw conclusions with regard to the various pathways that are affected between the two groups. Nevertheless, results obtained from comparing E+ rats under TN versus HS

conditions revealed that E+ diet intake under HS conditions down-regulated various genes associated with ATP synthesis, immune function, chaperone activity, and antioxidant function. Terminal dUTP nick-end labeling (TUNEL) positive hepatic nuclei and CYP3A4 protein expression increased in E+HS with a reduction in SOD and GPx activities. The present findings suggest that rats respond to E+ during short-term HS by inducing hepatic CYP expression and suppressing chaperone, antioxidant, and immune systems, which could ultimately increase cellular stress, resulting in various pathological abnormalities.

In the final study, rats were used to evaluate the effect of endophytic toxins on hepatic gene expression under long-term (three weeks) TN and HS conditions. Both E+ and HS induced alterations in hepatic gene expression were evaluated using DNA microarrays. Rats (n = 24) were fed an E+ diet and maintained at TN (21°C) for five days, followed by TN or HS (31°C) conditions for three weeks. Long-term intake of an E+ diet under TN conditions (E-TN versus E+TN) up-regulated genes coding for immune and electron transport functions, but down-regulated genes associated with antioxidant, xenobiotic, and chaperonic activities. Long-term intake of an E+ diet under HS conditions (E-HS versus E+HS) down-regulated genes coding for immune system, as well as carbohydrate, lipid, and protein metabolisms. Chronic HS (E-TN versus E-HS) in the present study down-regulated genes associated with cell proliferation, detoxification, ATP synthesis, and immune function. The E+ treatment under long-term HS up-regulated genes coding for apoptosis and detoxification, but down-regulated genes associated with immune and antioxidant functions, as well as carbohydrate, lipid and protein metabolisms. Taken together, results from the present study revealed that long-term HS

increased the susceptibility of rats to oxidative stress. In addition, E+ intake further affected the animals' antioxidant and immune systems as well as chaperone activity, making the animals more susceptible to oxidative and other stressors.

The present findings identified partial adaptation of rats to long-term E+ diet under TN conditions and this adaptation could be attributed to increased gene expression for detoxification and immune response. However at HS, rats did not exhibit similar adaptation to long-term E+ diet and this could be attributed to decreased antioxidant and immune response associated genes. Down-regulation of these pathways could contribute to distress often observed in E+ diet fed animals under heat stress conditions.

CHAPTER I

INTRODUCTION

Fescue toxicosis is an economically important syndrome with estimated annual losses exceeding \$600 million in the United States alone (Hoveland et al., 1993). Within the United States, more than 8.5 million cattle graze on tall fescue, and more than half of the tall fescue forage is infected by the endophyte *Neotyphodium coenophialum* (Jones et al., 2004). The endophyte maintains a symbiotic relationship with the plant by improving its drought and heat tolerance (Malinowski et al., 2005) but produces toxins (ergopeptine alkaloids), which interact with dopaminergic, adrenergic and serotonergic receptors and reduce the heat tolerance, growth, and performance of cattle grazing on the endophyte-infected tall fescue grass (Cross et al., 1995; Oliver, 2005). Other species, including horses (Cross et al., 1995), sheep (Gadberry et al., 2003), rabbits (Filipov et al., 1998), rats (Spiers et al., 2005a), mice (Varney et al., 1991), meadow voles (Conover, 1998) and birds (Zavos et al., 1993) exhibit some of the clinical manifestations of fescue toxicosis that are seen in cattle.

The economic losses associated with heat stress (HS) in livestock industry are mainly because of its negative effects on feed intake, activity, daily gain, male and female reproductive performance, embryo development, etc. (Collier et al., 2006). As a result of reduced feed intake and direct HS effects, numerous changes occur in the digestive tract, acid-base chemistry, and hormone activity (West, 2003). Heat stressed animals often reduce feed intake and seek shade, as well as increase respiratory, sweating

and peripheral blood flow rates at the expense of blood flow to mammary gland (in case of dairy cows) (West, 2003). This result in reduced productive efficiency of the animals. Heat stress also decrease serum calorogenic hormones (T3 and T4) and alkaline phosphatase (El-Nouty et al., 1990), cholesterol (Abeni et al., 2007), immune response (Kelley et al., 1982) and increase serum prolactin (Smith et al., 1997) and oxidative stress (Salo et al., 1991). Annual economic losses due to HS alone for the US livestock industry have been estimated between \$1.69 to 2.36 billion dollars (St-Pierre et al., 2003). The dairy industry experiences the greatest economic losses (\$897 to \$1,500 million dollars) due to HS, followed by beef industry (\$370 million dollars), swine industry (\$299 to \$316 million dollars), and poultry industry (\$128 to \$165 million dollars) (St-Pierre et al., 2003).

It has been noted that many of the animal responses to ergopeptine alkaloids depend on ambient temperature (T_a), with augmented hyperthermic and hypothermic conditions observed in animals fed E+ diets in hot and cold environments, respectively (Neal and Schmidt, 1985; Osborn et al., 1992; Spiers et al., 1995). Injection of rats with ergovaline (EV), the primary ergopeptine alkaloid found in endophyte-infected fescue, (Zhang et al., 1994) results in hypothermia at TN and hyperthermia under HS conditions. Other signs often observed with intake of E+ diet include decreased feed intake (FI), growth rate and serum prolactin and these signs are independent of T_a . The combination of altered thermoregulatory ability and reduced caloric intake over an extended period of time will likely have a negative impact on general animal health and performance.

Like cattle, rats fed a diet containing endophyte-infected fescue seed (E+) exhibit reductions in average daily FI and growth (Spiers et al., 2005a), making the rat a useful

animal model for the study of fescue toxicosis under controlled environmental conditions (Osborn et al., 1992; Spiers et al., 2005a).

The liver is the most important organ for xenobiotic metabolism and detoxification, and has been identified as a target organ of fescue toxicosis (Oliver, 1997). Long-term intake of E+ diet in cattle increases liver-specific enzymes (Piper et al., 1991), and a component of the fescue toxicosis response in rats is reduced liver weight (Settivari et al., 2006). Chestnut et al. (1992) noted that this reduction in liver weights in rats fed an E+ diet is also observed in rats fed a control diet at the same caloric level (i.e., pair-fed group). Recently, Bhusari et al. (2006) reported that E+ mice differentially express hepatic genes coding for cholesterol biosynthesis, antioxidants and *de novo* lipogenesis under TN and HS conditions.

Little experimental data is available for a detailed comparative evaluation of the effects of ergopeptine alkaloids on these research endpoints under controlled conditions of short- and long-term exposures to heat, and such information would facilitate the characterization of adaptation to one or both of these stressors (*i.e.*, ergopeptine alkaloids and/or heat).

The first objective of the present study was to evaluate the temporal effects of E+ and/or HS on various physiological responses, including core temperature (T_c), activity count, FI, feed conversion efficiency (FCE), average daily gain (ADG), selected serum biochemical parameters and visceral organ weights. The second objective was to identify the temporal effects of E+ and/or HS on hepatic gene expression and to identify various pathways that are affected as a result of either of the two stressors or the combination. It was hypothesized that there are temporal differences in physiological and genomic

responses to tall fescue endophytic toxins under thermoneutral, short-term and long-term heat conditions that characterize potentially adaptive processes.

CHAPTER II

LITERATURE REVIEW

Physiological responses associated with fescue toxicosis are often discussed in context of the interaction with Ta. Since toxins associated with fescue toxicosis alter thermoregulatory ability of the exposed animals, this review will discuss the normal mechanisms of thermoregulation, and the action of ergot alkaloids in different thermal environments. Then, the effects of fescue toxicants on hepatic detoxification pathways will be discussed. These include cytochrome P450, antioxidants, and drug efflux proteins. Finally, a brief description of microarray technique will be presented.

1. HEAT STRESS

1. 1. *Introduction*

A majority of the world's domestic animal populations are in regions where environmental stressors adversely influence productive efficiency (Gaughan et al., 1999). Annual economic losses due to HS alone for the US livestock industry have been estimated between \$1.69 to 2.36 billion (St-Pierre et al., 2003). The dairy industry experiences the greatest economic losses (\$897 to \$1,500 million) due to HS, followed by beef (\$370 million), swine (\$299 to \$316 million), and poultry (\$128 to \$165 million) industries (St-Pierre et al., 2003).

1. 2. *Effects of heat stress on livestock performance*

The economic losses associated with HS in livestock industry are mainly because of its negative effects on feed intake, activity, daily gain, male and female reproductive

performance, embryo development, etc. (Collier et al., 2006). As a result of reduced feed intake and direct HS effects, numerous changes occur in the digestive tract, acid-base chemistry, and hormone activity (West, 2003). Heat stressed animals often reduce feed intake and seek shade, as well as increase respiratory, sweating and peripheral blood flow rates at the expense of blood flow to mammary gland (in case of dairy cows) (West, 2003). This would result in reduced productive efficiency of the animals.

1. 2. 1. Effects on respiratory rate and animal core temperature

Animals under HS conditions increase both respiratory rate and water intake, and reduce feed intake and serum T3 (reduced serum T3 results in reduced metabolic heat production) levels to maintain homeothermy (Pereira et al. 2007). In conditions where these avenues of heat loss and production fail to maintain homeothermy, animals experience hyperthermia. Also, with increased respiration rate, a marked decline in blood carbon dioxide (CO₂) to oxygen (O₂) partial pressure ratio occurs, leading to respiratory alkalosis (Dale et al., 1954). This results in elevated blood and urine pH and decreased net acid excretion rate (Sanchez et al., 1994). Respiratory alkalosis associated with HS also disturbs ruminal microfloral environment, contributing to the overall deleterious effects on production and physiological status of the animal (Wallace et al., 1996).

Elvinger et al. (1992) exposed dairy cows to hot environments (average black globe temperature = 41°C) for 15 days. They observed increased rectal temperature and respiration rate in heat stressed cows compared to the cows maintained at TN throughout the study period. Pereira et al. (2007) observed greater rectal temperature in steers that were less resistant to HS (36°C for 13 days). Srikandakumar et al. (2004) compared the rectal temperature and respiration rate in black wooly Omani and white wooly Australian

Merino sheep during December (temperature humidity index; THI = 72 ± 1.4) and July (THI = 93 ± 3.1) months. They observed increased rectal temperature and respiration rate in both species during July compared to December.

1. 2. 2. *Effects of heat stress on feed intake and daily gain*

Most of the responses to HS are aimed at maintaining normal T_c. It is widely known that HS reduces feed intake in an attempt to reduce the body heat, generated by ruminal fermentation and body metabolism (Sanchez et al., 1994). Under chronic stressful conditions, animals lower food intake together with blood circulating thyroid hormone levels resulting in lower metabolic rate (Pereira et al., 2007). Typically, other factors such as relative humidity, wind velocity, radiation load, and feed quality also interact to affect the amount of feed consumed by a heat stressed animal under field conditions (Sanchez et al., 1994).

Heat stress also reduces digestive tract motility, ruminal contraction rates, ingesta passage rates, and increases gut mean retention time (Sanchez et al., 1994). Heat stressed (32°C) cows have greater digestibility of diets compared to those at TN (18°C) conditions (Warren et al., 1974). Cows with shade and without shade when fed the same amount of diet, cows without shade exhibited increased ruminal acid production per unit of fermentable feed, and their ruminal pH decreased compared to the cows in shade, contributing to greater digestibility of feed under heat (Niles et al., 1980). However, Wallace et al. (1996) reported a significant depression in body weight gain and feed to gain conversion in heat stressed beef calves. Brosh et al. (1998) fed heifers either high or low metabolizable energy (ME) diets during morning or evening hours under HS (30°C) conditions, with or without shade. Heifers consumed more O₂ when fed the high ME diet

in morning versus evening hours, irrespective of shade conditions. Furthermore, the high ME diet intake resulted in greater O₂ consumption than the low ME diet, more so in the morning than in the evening. This study highlights the significance of feeding high ME during cool periods rather than in the morning, in order to enhance thermal status of the animal.

Pereira et al. (2007) studied the effects of thermal stress (36°C for 13 days) on Tc and feed intake response in four different cattle breeds (Alentejana, Frisian, Mertolenga and Limousine breeds). They observed least feed intake changes in the heat resistant breeds (Mertolenga and Frisian), which suggests that the reduction in feed intake under HS depends on the level of strain the animal underwent. Others have shown that feed intake in rats is reduced with an exposure to HS (Johnson and Strack, 1989; Spiers et al., 2005a). Reduced feed intake in the HS rats (32°C) contributes to reduced average daily gain (Johnson and Stack, 1989; Spiers et al., 2005a).

1. 2. 3. *Effects of heat stress on hormones and blood chemistry*

Serum chemistry provides information about carbohydrate, lipid, protein, mineral metabolism, as well as other aspects of normal and abnormal physiology. Clinical signs often observed in HS animals include decreased blood calorogenic hormones (i.e., T₄, T₃) and alkaline phosphatase (Mitra et al., 1972; El-Nouty et al., 1990), but increased prolactin (PRL) levels (Smith et al., 1997). Cattle exhibit decreased T₃ and T₄ concentrations (33 and 15%, respectively) during summer months but increase concentrations during winter months (Johnson and Vanjonack, 1976). Reduced thyroid hormone levels under HS conditions are due to the complex neuroendocrine processes related to interactions among thermosensors, hypothalamus, pituitary and thyroid glands

(Pereira et al., 2007). Furthermore, hyperthermia inhibits the TSH-releasing hormone (TRH) secretion by hypothalamus, resulting in decreased thyroid activity (Pereira et al., 2007). A positive correlation is known to exist between Ta and serum PRL concentrations. Serum PRL concentrations in steers rise when their Ta is raised from 20 to 40°C, and their concentrations decrease when their Ta is reduced from 20 to 4°C (Smith et al., 1977). Similarly, Wetteman and Tucker (1976) observed elevated PRL levels in heifers at 32°C, but reduced levels at 4.5°C compared to the animals maintained at 21°C. Heat stress exposure increases circulating PRL levels in rats (Neill, 1970), goats (Sergent et al., 1988), sheep (Salah et al., 1995), and poultry (Johnson, 1981). Prolactin is known to stimulate water and mineral intake in lower vertebrates (Bern, 1975), rabbits (Burstyn, 1978), rats (Kaufman et al., 1981) and humans (Horrobin et al., 1971). Therefore increased prolactin levels under HS conditions, help the animals to maintain water and salt balance and at the same time to regulate their body temperature.

Catecholamines (epinephrine and norepinephrine) play an important role in thermoregulation, and their plasma levels may be used as an index of thermal stress (Davis et al., 1984). Davis et al. (1984) evaluated the effects of short-term HS (32 or 42°C for 8 hours) or cold exposure (13°C) on blood catecholamines (blood catecholamine levels increase with stress) in Longhorn and Hereford cattle. They observed that HS increased catecholamine levels only in Hereford during heat conditions as Longhorn breeds were heat tolerant. Similarly, they observed elevated levels of catecholamines only in Longhorn during cold conditions as Hereford cattle are cold tolerant. Similarly, Alvarez and Johnson (1973) observed elevated plasma and urinary norepinephrine levels of non-lactating Holstein during heat exposure (35 to 37°C).

Heat stress is known to have negative effects on serum cholesterol level. Abeni et al. (2007) monitored serum cholesterol level in dairy cows under summer conditions. They observed reduced plasma cholesterol concentration in heat stressed cows. A similar reduction in serum cholesterol level was evident in rats under HS (Fuquay et al., 1980). Three-week-old chicks exposed to HS (38°C for 2 hours/day) for 6 days resulted in reduced serum cholesterol level (Zulkifli et al., 2000).

Heat stress reduces serum alkaline phosphatase (ALP) level in different species. Abeni et al. (2007) observed reduced plasma ALP activity in dairy cows during summer months and they confirmed that this enzyme as a quick and reliable blood-marker for HS. Niu et al. (2007) observed a similar reduction in serum ALP, but increases in serum creatinine, urea nitrogen, aspartate aminotransferase, alanine aminotransferase in rats exposed to 43°C for 20 min.

1. 2. 4. *Effects of heat stress on immune system*

Chronic heat is known to compromise immune status of the animal. Kelley et al. (1982) exposed Holstein calves to constant HS (35°C) or TN (23°C) conditions and measured their immune responses at different time periods (i.e., at the end of 3, 7 and 14 days). They observed that after HS for 3 to 14 days, immunoglobulin G1 averaged 27% less in HS calves than in calves maintained at TN. Elvinger et al. (1992) exposed dairy cows to HS (average black globe temperature = 41°C) for 15 days. They observed greater packed cell volume (possibly due to shock or dehydration) in HS cows than for cows at TN. They also reported that the migrations of leukocytes to the mammary tissue, in response to a chemotactic challenge of oyster glycogen, were reduced under HS conditions. In a previous study, the same group observed that HS (42°C) inhibited

random migration and chemotaxis of polymorphonuclear leukocytes compared to cows maintained at TN. Joseph et al. (1991) studied the effects of acute HS (40°C for 30 minutes) on immunological parameters in male albino rats. They observed decreased total WBC count and neutrophilia, eosinophilia and lymphocytopenia in HS rats compared to controls maintained at TN. They also noticed an increase in phagocytic index and lymph node/BW, but a reduction in avidity index, thymus and spleen weight/BW. These results show that HS has detrimental effects on immune status of an animal.

1. 2. 5. Role of heat stress in inducing oxidative stress

Heat stress causes protein structure instability throughout the body leading to deleterious denaturation of various essential proteins (Salo et al., 1991). Several reports in the past have correlated HS and hyperthermia with increased levels of oxidative stress (Salo et al., 1991).

Sub-lethal H₂O₂ exposure in *Saccharomyces cerevisiae* elicits the induction of a variety of heat shock response genes involved in thermo protection, including heat shock protein (HSP) 104 (involved in solubilization of aggregated proteins and the protection of mRNA splicing), HSP 70 (protein folding and refolding), as well as the antioxidant enzymes catalase, thioredoxin peroxidase and cytochrome C peroxidase (Godon et al., 1998). Heat exposure provides cross-tolerance to lethal H₂O₂ exposure and depletion of protein antioxidant defenses, including catalase, cytochrome C peroxidase, superoxide dismutase and thioredoxin peroxidase sensitizes cells to lethal heat exposures (Davidson et al., 2006). Niu et al. (2007) observed increased levels of lipid peroxidation and oxidized-form glutathione/reduced-form of glutathione ratio in hypothalamus in rats exposed to HS (43°C for 20 min.) suggesting oxidative stress.

Zhang et al. (2003) tested if HS would increase the hepatic free radical levels in two-year- and six-month-old rats. They heated the rats using an infrared lamp until T_c reached 41°C and then maintained them at this HS level for 30 minutes. Following HS, the rats were allowed to passively cool at room temperature. A second HS exposure occurred 24 hours after the first. After the second HS, the rats were euthanized at various time points (0, 2, 6, 12 and 24 hours) and liver sections were collected. They followed a similar protocol for the sham groups, except they were maintained at 22 to 24°C throughout the experiment. They observed an increase in the oxidative fluorescent markers dihydroethidium and dihydrofluorescein diacetate in both age groups. In six-month-old rats, reactive oxygen species (ROS) levels increased only in the early stages after heating (1, 2 and 6 hours), returning to control level by 12 hours; however in two-year-old rats, the marker levels remained high throughout the 24 hour post-stress period. They also observed hepatocellular oxidative damage to lipids and DNA in two-year-old rats at all time points. The antioxidant levels of glutathione (GSH) to glutathione disulfide (GSSG) was reduced in heat stressed rats; however, in six-month-old rats, the levels returned to TN level by 12 hours post-stress, whereas in two-year-old rats, the levels remained low even at the end of 24 hours post-stress. Their study proved that hyperthermic challenge produces oxidative stress in rat hepatic tissue with increased levels of reactive oxygen species production and reduced antioxidant enzyme levels.

Bernabucci et al. (2002) measured antioxidant enzyme levels in transition cattle exposed to mild HS (rectal temperature = $39.5 \pm 0.2^\circ\text{C}$; THI = 73.2 ± 2.5) conditions during April to August. They did not observe any difference in plasma glutathione peroxidase (GPx), thiol groups (SH), reactive oxygen metabolites, or thiobarbituric acid

reactive substrates (TBARS) between spring (April to May) and summer cows (June to August). However, they observed increased levels of erythrocyte superoxide dismutase, GPx, SH, and TBARS in summer cows compared to spring cows, indicating a condition of oxidative stress in summer transition dairy cows. They hypothesized that during HS, increased blood O₂ pressure, due to increased respiratory rate, might be the cause of alterations of oxidative status of erythrocytes. They also hypothesized that the presence of high concentrations of polyunsaturated fatty acids in erythrocyte membranes, and the presence of high concentrations of iron in hemoglobin might generate free radicals, which could in turn cause alterations in erythrocyte antioxidant defense systems. They reported that the increased antioxidant enzyme levels in summer cows represent compensatory changes that the cows undergo in response to oxidative stress. They suggested that erythrocytes are an appropriate and sensitive model to study the oxidative status of dairy cows in hot environments.

Harmon et al. (1997), in contrast, reported the reduction of plasma antioxidant activity in mid lactating cows exposed to a chamber temperature of 29.5°C. Bernabucci et al. (2002) did not observe any difference in the concentrations of lipid soluble antioxidants, i.e., α -tocopherol, β -carotene, retinol and retinyl palmitate in muscle tissue during 10 day continuous heat (mean dry bulb temperature = 38.3°C). Bernabucci et al. (2002) suggested that the discrepancies observed between such studies are mainly due to the differences in the physiological status of the experimental animals and the tissue tested across the studies.

Free radicals at low levels can induce antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Klatt and Lamas, 2000). However, when

free radicals are in excess of the antioxidant capacity they in fact become inhibitory to these enzymes and factors (Klatt and Lamas, 2000). Antioxidant vitamins, such as E and C, act as scavengers of free radicals to minimize DNA damage by glycooxidation (Arechiga et al., 1998).

1. 2. 6. *Effects of heat stress on heat shock proteins*

Most animals react to heat and other stressors by synthesizing the evolutionary-conserved HSP (Wu, 1995). They are classified based on their molecular weights. The major ones are HSP 150, HSP 110, HSP 90, HSP 70, HSP 60, HSP 40 (Malago et al., 2002).

Under stressful conditions, a family of DNA-binding proteins called heat shock factors (HSF1-4, of which the best known is HSF1) transactivates various HSPs (Malago et al., 2002). In unstressed cells, the inactive HSF is bound to the cytoplasmic HSP40, 70 and 90 in monomeric form without the DNA-binding activity. In response to stress, HSF is released and translocates into nucleus, where it assembles into a trimer and binds to a heat shock regulatory element present in the heat shock gene promoter. This binding results in transcriptional activation of various HSPs (Santoro, 2000). The resulting HSPs act as molecular chaperones and enhance cellular stability in an attempt to cope with the severe conditions, thereby enabling the cell structure to function optimally and survive the impact (Horowitz, 1998).

Up-regulation of HSPs as a result of whole body hyperthermia (42°C for 15 min) protects myocardial tissue against subsequent ischemia and hyperthermic injury in the rat (Yamashita et al., 1998). Li et al. (2001) reported that HSPs generated through HS (45°C for 60 minutes) confer cardiovascular protection during heat stroke by alleviating severe

hypotension and bradycardia by potentiating the sensitivity and capacity of baroreceptor reflex response.

The HSPs provide thermo-tolerance in animals by preventing protein denaturation or increasing its repair (Kregel, 2002). By virtue of their anti-oxidant actions, HSPs sequester pro-oxidant free iron, thereby reducing oxidative stress within cell (Sonna et al., 2002). Sensitive organs such as the liver and small intestine are the first to accumulate HSPs in response to whole body hyperthermia (Flanagan et al., 1995). Furthermore, HSPs are known to protect against different kinds of environmental stressors such as infection, inflammation, toxins, ultraviolet light, starvation, etc. (Malago et al., 2002).

1. 2. 7. Short versus long term heat stress effects on animal performance

Many animal studies have concentrated on the effects of acute (30 to 120 min.) and intense HS (41 to 42°C) on physiological parameters. However, in real world, animals are often exposed to chronic (days to months) and less intense (33 to 36°C) HS (Jordan, 2003; St-Pierre et al., 2003). Exposure to stressful conditions initially results in impaired cellular performance (Schwimmer et al., 2006). To compensate for it, the short-term heat acclimation (STHA) process increases excitability of autonomic nervous system outflow signals. This is followed by enhanced cellular functions during long-term heat acclimation (LTHA) that is accompanied by decreased autonomic excitability (Schwimmer et al., 2006). During LTHA, hypothalamic threshold for body temperature is decreased, resulting in activation of heat-dissipation mechanisms. Furthermore, the body temperature threshold for the development of thermal injury increases under this condition (Schwimmer et al., 2006).

Schwimmer et al. (2006) continuously exposed rats to $34 \pm 1^\circ\text{C}$ for 2 days (STHA) or for 30 days (LTHA) to identify the hypothalamic gene expression changes in response to acute or chronic HS. They observed an up-regulation in genes coding for voltage-gated ion channels, ion pumps or transporters, hormone or transmitter receptors, and cellular messengers leading to enhanced neurotransmitter signaling. They observed a down-regulation of genes associated with intracellular protein trafficking, metabolism, and phosphorylation processes to imply a perturbation in cellular maintenance. During LTHA, genes encoding ion movement and cellular signaling returned to normal levels but genes encoding mitochondrial energy metabolism, immune response, and cellular maintenance processes were up-regulated. These results represent a shift from an early transient, perturbed state (STHA) to a long-lasting efficient and stable acclamatory homeostasis (Schwimmer et al., 2006).

Rats are known to adapt to chronic HS by decreasing metabolic heat production (Arieli and Chinet, 1986; Shido et al., 1991; Gordon, 1993) and increasing heat loss activities (Horowitz et al., 1983). Horowitz et al. (2004) continuously exposed rats to $34 \pm 1^\circ\text{C}$ for 2 d (STHA) or for 30 d (LTHA) to identify the cardiac gene expression changes in response to HS, and its association to ischemic-reperfusion cross-tolerance. At the end of 2 d or 30 d period, the rats received a thermal challenge by exposure to 41°C for 2 hours. They observed an increase in the expression of genes coding for DNA damage/repair, free radical scavenging, stress regulation, and effectors to protect the cells from DNA damage and oxidative stress. However, they did not observe the up-regulation of these genes in the chronic study. Instead, they observed the up-regulation of genes coding for adaptive and long-lasting adaptive pathways, such as DnaJ (HSP40)

homologs (required for stabilizing the interaction of HSP 70), anti-apoptotic genes, and anti-oxidants. These results suggest that the physiological and genomic responses to HS vary temporally in a selective manner.

2. FESCUE TOXICOSIS

2. 1. *Introduction – Fescue toxicosis*

A large portion of the pasture grass in the US is tall fescue (*Festuca arundinacea*), and it is considered as the primary cool season grass for livestock (Sleper and Buckner, 1995). Tall fescue is reported to be imported into US from Europe as early as 1771 (Terrell, 1979). It is known for its tolerance to drought, insects, and extreme soil pH (4.7 to 9.5; Cowan, 1956). This grass has high seed production and forage yields, vigorous rooting, and a long grazing season (Hanson, 1979).

Low demand on labor and grazing control make tall fescue a highly favorable cultivar for livestock producers. Favorable environments for tall fescue grass include humid and moderately temperate zones (Burns and Chamblee, 1979). Tall fescue is expected to occupy greater than 14 million hectares in the US, extending from northern Florida to Canadian border. Nutritive value of tall fescue (100 to 200 g crude protein/kg) depends on genetic variant, plant maturity, and soil fertility. Highest nutritive value is observed during the fall due to both high carbohydrate solubility and low lignin content (Brown et al., 1963) and lowest during summer (Bughrara et al., 1991).

2. 2. *Symbiotic association of fescue with endophyte*

An endophytic fungus (*Neotyphodium coenophialum*) grows within tall fescue and has a mutual symbiotic relationship with the grass. The endophyte grows between the

cell walls of the tall fescue, with mycelia growing throughout the intracellular spaces (Kimbrough et al., 1993). It does not invade the plant cells nor is it pathogenic to the plant (Thompson et al., 2001). The endophyte infects the developing seed and its life cycle follows that of the fescue grass. The fungus and fescue grass interaction produces various ergot alkaloids, which are toxic to grazing animals.

Fescue grass benefits from the endophyte-grass association, in part, because of a group of ergopeptine alkaloids produced by the endophyte or the plant in response to the endophyte. Ergopeptine and clavine alkaloids, peramine and loline alkaloids, are biologically active and inhibit the activities of root eating nematodes and other insects, and also reduce reproduction and growth in large animals (Thompson et al., 2001). Fescue toxicosis is not just a problem for livestock, but also affects other species, including horses (Cross et al., 1995), sheep (Gadberry et al., 2003), rabbits (Filipov et al., 1998), rats (Spiers et al., 2005a), mice (Varney et al., 1991), meadow voles (Conover, 1998) and birds (Zavos et al., 1993).

The association also reduces plant defoliation, which helps the plant to capture solar energy (Thompson et al., 2001). Endophyte-infected tall fescue has greater forage and seed productivity than the uninfected (E-) form (Hill et al., 1991) and is more drought and insect tolerant (West et al., 1993). The endophyte, in turn, receives a home and food supply and therefore it is never subjected to environmental forces and is only dependent upon plant health for its survival (Hill, 1994). So, it is in the plant's interest to provide the needs for the endophyte; and the endophyte to provide protection to the plant against climatic and biological forces (e.g., insects) that may threaten the plants.

The *Neotyphodium* genus, unlike the *Claviceps* genus (similar fungi which produce ergotism) do not transmit from one plant to another, but rather are confined to the seed and transmit only through seeds (Sleper and Buckner, 1995). In a survey of over 1,500 pasture samples tested throughout US, more than 70% of the samples had 60% or more endophyte infection rate (Shelby and Dalrymple, 1987).

2. 2. 1. *Toxins associated with fescue toxicosis*

Some of the ergot alkaloids associated with endophyte infected tall fescue (E+) include ergovaline, ergotamine, ergocryptine, ergocornine, ergosine, lysergic acid amide, and ergonovine (Porter, 1994). Ergopeptides are non-ribosomally-synthesized peptides, containing lysergic acid and three amino acids that vary between and define the molecules in the family (Panaccione et al., 2001). Ergovaline has historically been identified as the putative toxin causing fescue toxicosis in E+ grazing livestock (Oliver, 1997; Porter, 1994). It comprises more than 85% of total ergopeptide alkaloids found in E+ (McCollough et al., 1994). Ergovaline contains D-lysergic acid, L-alanine, L-valine and L-proline and differs from ergotamine, the most common ergopeptide, in containing L-valine in place of L-phenylalanine (Panaccione et al., 2001). Peramine is another ergot product produced by the fungus-grass interaction in infected tall fescue grass. Peramine alkaloids cause minimal toxicity in mammals (Cheeke, 1995; Porter, 1995), but are toxic to insects (Porter 1994). However, some insects adapt to peramine and use it for their own protection against predators (Porter, 1994). Loline alkaloids, though present in high concentrations in E+, have little effect on grazing animals (Fletcher et al., 2000; Gadberry et al., 2003) but are toxic to many insects (Wilkinson et al., 2000). Although loline alkaloids alone are not thought to cause fescue toxicosis; Gadberry et al. (2003)

hypothesized a potential synergistic action with ergopeptine alkaloids that might contribute to the condition.

2. 2. 1. 1. *Ergopeptine versus ergoline alkaloids*

Although ergopeptine alkaloids are considered to be responsible for majority of clinical signs associated with fescue toxicosis, some researchers (Hill, 2005) have proposed that ergoline alkaloids play a major role in producing the syndrome. Hill et al. (2001) reported ergoline alkaloid (lysergic acid) is a circulating metabolite of ergot alkaloid metabolism in herbivores. Lysergic acid was shown to have a greater absorption potential across ruminant foregut *in vitro* than ergopeptines. Oliver et al. (1993) has shown that a lysergic acid analog, lysergic acid amide, has a vasoconstrictive activity in bovine tissue. Similarly, various ergopeptine alkaloids, such as ergotamine (Solomons et al., 1989) and ergovaline (Dyer, 1993) vasoconstrict blood vessels in cattle. However, Klotz et al. (2006) reported that lysergic acid is a weak vasoconstrictor and its vasoconstrictor properties are evident only at very high levels (1×10^{-4}) (approximately 7-fold the maximum concentration often found in E+ grass). In contrast, Dyer (1993) observed that bovine uterine and umbilical veins exposed to low concentrations of ergovaline (1×10^{-8}) exhibit a slow and prolonged vasoconstriction mediated through serotonin receptors. It also has a greater potential for bioaccumulation than lysergic acids. Likewise, various *in vitro* (Strickland et al., 1994) and *in vivo* (Spiers et al., 1995) studies using pure ergovaline support the hypothesis that ergovaline contributes to many of the clinical signs associated with fescue toxicosis. Spiers et al. (1995) reported hyperthermia in heat stressed (31 to 33°C) rats' injected intraperitoneally with ergovaline, as a result of reduced peripheral heat loss across the tail. Other livestock maladies associated with

ergovaline consumption include reduced feed intake, poor weight gain, and hormonal imbalances leading to reduced fertility, reduced lactation, and gangrene of limbs (Panaccione et al., 2001). Therefore it can be speculated that ergoline compounds by themselves are very weak vasoconstrictors, and therefore do not have the capability to cause fescue toxicosis by themselves. However, in combination with ergopeptine alkaloids, they might contribute to the clinical signs associated with fescue toxicosis.

Ergovaline may not be the only contributor to fescue toxicosis and may work in concert with other ergot alkaloids to produce the toxicological syndrome (Gadberry et al., 2003). Gadberry et al. (2003) tested lambs to determine if a diet containing synthetic ergovaline and a diet containing E+ seed would exhibit similar clinical signs. Their diets consisted of 10% E- fescue seed diet (E- group), 10% E- fescue seed diet containing pure EV (EV group), and 10% E+ fescue seed diet (E+ group). They observed a reduction in feed intake and growth rate of the E+ group compared to both E- and EV groups, with no difference between EV and E- groups. The EV did cause peripheral vasoconstriction and reduction in serum PRL levels compared to E- group, however, the E+ diet produced a greater response compared to EV diet alone. Therefore, they concluded that ergovaline is a fescue toxicant; however, it is not as effective as the E+ diet in producing fescue toxicosis symptoms, to suggest that additional alkaloids might contribute to the full fescue toxicosis response.

2. 3. Toxicological conditions associated with fescue toxicosis

Physiological responses to fescue toxicosis are often discussed in the context of an interaction with Ta. Fescue toxicosis-related health problems are exacerbated by extreme Ta. The type of thermal stress (i.e., cold versus hot) experienced by livestock

exposed to E+ tall fescue is an important determinant of which clinical signs are observed with fescue toxicosis (Hemken et al., 1981). The toxicological conditions associated with fescue toxicosis can be broadly divided into three types. They include summer slump, fescue foot and fat necrosis.

During HS conditions, an economically significant syndrome (i.e., summer slump) occurs in E+ fed animals, which results from alterations in thermoregulatory ability and changes in endocrine and neurotransmitter milieus (Burrows and Tyrl, 2001; Kerr and Kelch, 1999; Paterson et al., 1995). The E+ beef cattle under HS conditions, exhibit poor performance due to disruption of the normal patterns of diurnal T_c (Al-Haidary et al., 2001; Burke et al., 2001). Summer slump is more common in cattle and sheep, under hot and humid conditions, and it is characterized by hyperthermia, rough hair coat, tachypnea, increased salivation, hypoprolactinemia, and reductions in reproductive performance. Feed intake, growth rate and milk production (Aldrich et al., 1993; Burrows and Tyrl, 2001; Kerr and Kelch, 1999) may also be reduced, but this may occur at any T_a.

In contrast to summer slump, fescue foot occurs in E+ fed animals during cold environmental temperatures and it is characterized by tissue necrosis and dry gangrene of the distal extremities, including tips of the ears and tail (Yates, 1983). Fescue foot develops in cattle during long-term intake of endophyte-infected tall fescue hay or pasture, and most commonly affects the hind limbs (Spiers et al., 2005b). Clinical signs start with swelling and reddening at coronary band, knuckling of the pastern joint, and arching of the back and in extreme cases. Animals will appear unthrifty, with increasing lameness and inflammation and ischemic necrosis of the hooves, associated phalanges,

joints and soft tissue (Burrows and Tyrl, 2001). Furthermore, under cold conditions, E+ fed animals' exhibit hypothermia. Spiers et al. (1995) reported hypothermia in rats following ip injection of ergovaline under TN and cold stress conditions, which they attributed to reduced metabolic heat production.

Both summer slump and fescue foot conditions occur mainly because of peripheral vasoconstriction, with the animal being unable to dissipate its internal heat during the summer and supply sufficient nutrients to active tissues during winter conditions. Fat necrosis is a rare condition characterized by formation of large necrotic fat masses within the stomach region, which obstruct gastrointestinal and reproductive tracts leading to various abnormalities (Smith et al., 2004).

2. 3. 1. Effects of endophyte-infected fescue on livestock performance

Livestock that graze endophyte-infected tall fescue diet (E+) develop clinical signs collectively known as fescue toxicosis (Gadberry et al., 2003). Characteristic clinical signs associated with fescue toxicosis vary with the level of environmental stress and thermal strain of the animal. Reduced feed intake and increased Tc are probably responsible for many of the problems associated with fescue toxicosis.

2. 3. 2. Effects of endophyte-infected fescue on core body temperature and respiratory rate

Fescue toxicosis-related health problems are Ta-dependent and are exacerbated by extreme conditions. The level of environmental stress experienced by livestock exposed to E+ tall fescue is an important determinant of which clinical signs are observed in cases of fescue toxicosis (Spiers et al., 2005b). Under cold or TN conditions, E+ fed animals exhibit lower or unaltered Tc, with no change in respiration rate. Neal and Schmidt

(1985) observed lowered Tc in rats fed an E+ diet 21°C for 15 days. Similarly, Spiers et al. (1995) observed a decrease in both Tc and tail temperature in ergovaline-injected rats at 7-9°C. However, Osborn et al. (1992) did not observe any change in Tc, as well as respiration rate, in steers consuming E+ diet at 21°C.

Intake of an E+ diet during HS increases Tc and respiration rate. Elevated Tc results from the inability to transfer core heat to peripheral tissues (Rhodes et al., 1991). This is because of peripheral vasoconstriction resulting from the agonistic properties of E+ toxicants with α -2 adrenergic and serotonin receptors (Oliver, 2005). Steers on an E+ diet exhibited only one-half the heat loss of steers consuming E- diet at 32°C, however, at 22°C heat loss was similar between E- and E+ animals (Paterson et al., 1995). Al-Haidary et al. (2001) reported that the exacerbated poor performance in E+ fed beef cattle during HS is associated with the disruption of the normal patterns of diurnal Tc. Similarly, Osborn et al. (1992) observed a significant increase in Tc and respiration rate in E+ diet fed steers at 32°C. Spiers et al. (1995) investigated the effects of injecting rats with ergovaline (EV) in hot environment (31 to 33°C). Treatment during HS resulted in an elevation of rectal temperature and a reduction in tail temperature suggesting peripheral vasoconstriction.

Nickerson (1970) and Loew et al. (1978) suggested that ergot alkaloids can increase Tc by a direct effect upon the temperature regulating areas in the brain. This central effect upon Tc was found to occur at a lower dosage compared to that necessary to mediate vasoconstriction (Thompson et al., 2001).

2. 3. 3. Effects of endophyte-infected fescue on feed intake

The effects of fescue toxicosis on feed intake have been extensively studied. A typical response to ingesting an E+ diet is a reduction in feed intake in both rodents (Spiers et al., 2005a) and livestock (Jackson et al., 1984). Neal and Schmidt (1985) observed decreased feed intake and growth rate in rats feeding on E+ diet at TN for 15 days. Similarly, Spiers et al. (2005a) observed feed intake reduction by more than 50% within the first 24 hours on E+ diet at TN. Likewise, cattle fed a diet containing E- seed at 22°C ate more than those fed a diet containing E+ seed (EV concentration in E+ diet was 285 ppb) (Aldrich et al., 1993).

The effect of E+ diet on feed intake appears to be exacerbated by HS (Hemken et al., 1981). At 32°C, Aldrich et al. (1993) observed greater feed intake of cattle on E- compared to E+ diets, though HS reduced feed intake for both groups. Similarly, Roberts et al. (2002) and Spiers et al. (2005a) observed decreased feed intake in rats fed an E+ diet under HS conditions. Likewise, Gadberry et al. (2003) reported a reduction in feed intake in lambs fed an E+ diet at 32°C compared to those on an E- diet.

The reason for the large, rapid reduction in feed intake is unknown. Spiers et al. (2005a) ruled out the possibility of amount of seed in the diet or palatability of the ground seed diet as the responsible causes for the reduction in feed intake, as rats fed on E- diet had identical content and amount of fescue seed.

Several neurotransmitters affect feed intake and gut motility. Ergot alkaloids possess high affinity for α -2 adrenergic, dopamine, and nearly all subtypes of 5-hydroxytryptamine (5-HT) receptors (Perz et al., 1999). Peripheral and central serotonin play an important role in normal satiation (Pollock and Rowland, 1981). Pollock and

Rowland (1981) observed a dose-related decrease in feed intake in hungry rats upon intraperitoneal serotonin injection. Similarly, injection of α -adrenergic receptor agonists into the paraventricular nucleus (Wellman et al., 1993) or intraperitoneal injection of dopamine agonist (Bednar et al., 1991) reduces feed intake in rats. Given the agonistic properties of ergot alkaloids with serotonin, dopamine, and adrenergic receptors, it is speculated that the reduced feed intake associated with fescue toxicosis could be partially associated with these receptors. Oliver et al. (2000) observed increased tryptophan levels in E+ fed cattle, and hypothesized that increased tryptophan levels impact serotonin levels in hypothalamus and thereby affect feeding behavior. Porter et al. (1990) reported increased serotonin metabolite levels in the hypothalamus of E+ fed cattle suggesting activation of serotonin receptors by E+ toxicants. Also, a majority of serotonin receptors are localized in gastrointestinal tract, where serotonin plays a major role in gut motility. Activation of serotonin receptors in gut may increase gut motility leading to impaired feed utilization and diarrhea, which are clinical signs often observed in E+ fed cattle (Coppock et al., 1989; McCollough et al., 1993).

Several attempts have been made to alleviate the clinical signs associated with fescue toxicosis. Dopamine antagonists such as haloperidol, phenothiazine, domperidone, metaclopramide, and spiperone have been tested to determine if they can minimize the toxicological conditions of fescue toxicosis. These antagonists increase serum PRL levels but have only limited effects on body heat dissipation (Bolt et al., 1983; Boling et al., 1989). Metaclopramide injections increase serum PRL and cholesterol levels, increase grazing time during the hot period of the day, increase daily gain, increase blood flow to the pancreas, adrenal gland and duodenum in steers (Lipham et al., 1989; Rhodes et al.,

1991) but do not alter dry matter digestibility or Tc in lambs (Aldrich et al., 1993). Intraperitoneal injection of prazosin (α -adrenergic antagonist) into rats fed an E+ diet increase dry matter intake and aid in the dissipation of body heat under HS conditions (Larson, 1993).

2. 3. 4. Effects of endophyte-infected fescue on body weight and growth rate

Decrease in BW and growth rate are characteristic symptoms of fescue toxicosis. The decrease in growth rate could be due to reduced feed intake and feed conversion efficiency. Spiers et al. (2005a) observed a parallel decrease in body weight and growth rate along with reduced feed intake in E+ fed rats at TN. They noted that the rate of increase in BW and ADG were less (70 to 78%) for E+ than for E- rats. Likewise, Neal and Schmidt (1985) observed a 63% decrease in ADG for E+ rats fed under similar conditions. Similarly, E+ fed cattle reduce ADG from 22 to 79% under a variety of ambient conditions (Schmidt et al., 1982; Stuedemann et al., 1986).

Under HS conditions, E+ diet fed animals are known to have a greater reduction in growth rate and BW. Gadberry et al. (2003) observed a greater reduction in average daily gain in E+ compared to E- lambs when they were fed with E+ feed under HS conditions. Similarly, Johnson and Stack (1989) reported that HS reduces BW and growth rate in rats. Spiers et al. (2005a) observed a similar decrease in average daily gain in rats when exposed to HS.

Prolactin is known to increase the circadian rhythm of lipogenic and insulin receptor numbers in hepatic tissue of hamsters, and inhibition of PRL secretion decreases hepatic lipogenic rates (Gerardo-Gettens, 1989). Similarly, Cross et al. (1995) suggested hypoprolactinemia as one of the main causes for decreased body weight in E+ fed cattle.

Therefore, hypoprolactinemia seen in E+ animals could contribute to the lowered BW gain in E+ rats by lowering hepatic lipogenic rates.

Clinical signs observed in fescue toxicosis may be due to the direct effects of toxicants or to reduced dry matter intake. To separate the reduced dry matter intake from reduced feed intake, Varney et al. (1991) used pair-fed studies, and identified developmental and pubertal delays in pup mice born to E+ seed fed dams. The E+ diets also increased estrous cycle abnormality and reduced testicular-epididymal weight. In a separate pair-fed study, Larson et al. (1992) observed that consumption of E+ seed by rats reduced dry matter intake by 11% and reduced growth and nitrogen (N) digestibility at TN (20°C). In the same study, exposure to HS (32°C) reduced dry matter intake, growth rate, body N gain, apparent N digestibility, and N retention. These observations support the notion that fescue toxicants by themselves reduce production efficiency in animals.

2. 3. 5. Effects of endophyte-infected fescue on feed conversion efficiency

The reduced feed conversion efficiency associated with fescue toxicosis could hinder the animal's ability to utilize the feed energy for production aspects. Diets containing E+ tall fescue were reported to reduce dry matter and organic matter digestibility in sheep (Hannah et al., 1990), cattle (Aldrich et al., 1993), and rats (Larson et al., 1992). Schmidt et al. (1982) noted a reduction in ADG in steers that consumed fescue toxins, and hypothesized that the observed reduction is not solely due to reduction in feed intake. Similarly, Osborn et al. (1992) observed that, even though some E+ fed steers consumed feed more than the calculated maintenance level (NRC, 1984) they lost weight. Reduction in ADG in the above cases could be mainly due to the reduction in feed conversion efficiency. Similar results were observed by others (Neal and Schmidt,

1985; Hoveland et al., 1993). The reasons for the reduction in feed conversion efficiency are not yet identified.

Dopamine and serotonin are capable of rapidly increasing gastric and small intestine motility and also vasoconstrict mesenteric blood vessels (Estan et al., 1995). Since it is known that ergot alkaloids act as dopaminergic and serotonergic agents, they activate the dopamine and serotonin pathways. This might result in improper feed energy absorption due to lack of sufficient nutrient absorption time (as a result of increased gastric and intestinal motility) resulting in the observed reduction in feed conversion efficiency. Thompson et al. (2001) concluded that growth in rats and cattle is affected by the endophyte in a manner beyond reduced intake, and they speculated that animals used the energy for other functions, such as hepatic mixed function oxidase activity related to detoxification rather than production activities. In support of this speculation, Zanzalari et al. (1989) observed an increase in sheep hepatic mixed function oxidase activity as a result of toxic fescue intake. Also, the effects of ergot alkaloids on various digestive enzymes, gastrointestinal tract epithelia, pH, and microflora are not known and this possibility cannot be ruled out since GIT is the first organ that is exposed to full spectrum of ergot alkaloids on oral intake. Although the reasons are not fully known, it can be concluded that fescue toxicosis impairs the animal's ability to fully utilize the feed energy for production purposes.

2. 3. 6. Interactions of ergot alkaloids associated with endophyte-infected fescue with biogenic amine receptors

Ergopeptide alkaloids, present in E+ tall fescue, affect various biogenic amine receptors (Dyer, 1993; Larson et al., 1999). Stimulation of these receptors by these

alkaloids contributes to vasoconstrictive properties that are known to deprive tissues of proper blood flow for heat loss and nutrition (Spiers et al., 2005a). Stimulation of α -2 adrenergic receptors by the alkaloids enhances blood platelet aggregation, which is likely involved in the various coagulopathies that occur with severe toxicity (Oliver, 1997).

Ergovaline has agonistic properties with α -2 adrenergic receptors and therefore has a greater effect on venous (where α -2 receptors predominate) compared to arterial locations (Oliver et al., 1993). Smaller blood vessels are more sensitive to ergot alkaloids compared to larger arteries (Oliver, 1997). Activation of α -2 adrenergic receptors often results in persistent vasoconstriction of smooth muscle cells in the veins (Oliver, 1997), which is a characteristic of fescue toxicosis. Dopamine-2 receptor (D_2) stimulation by these alkaloids reduces PRL secretion, which is the cause of the decreased milk secretion in animals that graze on E+ pastures (Larson et al., 1999).

Ergot alkaloids such as ergine and EV also possess high affinity for nearly all subtypes of 5-hydroxytryptamine (5-HT) receptors (Dyer, 1993). Several ergot alkaloids, including ergovaline and ergotamine have agonistic properties with serotonin-2 receptors (Oliver, 2005). Moreover, serotonin-2 receptors are present throughout the CNS, blood vessels, gastrointestinal tract, and on platelets (Martin, 1994). Activation of serotonin-2 receptors by EV results in both vasoconstriction and vascular smooth muscle cell proliferation leading to reduced vascular lumen (Oliver, 1997). Furthermore, various serotonin-2 receptor agonists such as 3, 4-methylenedioxymethamphetamine (MDMA) disrupt thermoregulatory ability of rats, making T_c dependent on the T_a above 26°C (Malberg and Seiden, 1998). Given the agonistic properties of ergot alkaloids associated with fescue toxicosis, it can be speculated that serotonin receptors also contribute to the

hyperthermic conditions often seen in E+ diet fed animals during HS. Cattle grazing on E+ tall fescue pasture have increased serotonin metabolites in the central nervous system, suggesting activation of serotonin receptors as a result of E+ diet intake (Porter et al., 1992). Oliver et al. (2000) observed increased tryptophan levels in serum from cattle on E+ pasture, to result in reduced feed intake (Rossi-Fanelli and Cangiano, 1991).

2. 3. 7. Effects of endophyte-infected fescue on blood chemistry and hormonal changes

Toxicants associated with fescue toxicosis affect a variety of enzymatic systems associated with the liver. Intake of an E+ diet reduces serum alkaline phosphatase, aspartate aminotransferase, and aniline transaminase (Piper et al., 1991; Thompson and Steudeman, 1993). Piper et al. (1991) hypothesized that the depression in transaminases in E+ fed animals is likely due to hepatic toxicity, which could depress overall hepatic detoxifying efficiency (Piper et al., 1991). However, Oliver (1997) and Schultze et al. (1999) suggested that the reductions in hepatic enzyme activity in E+ animals were due to nutrient deficiency (minerals, vitamins and proteins) as a result of reduced caloric intake.

Ergot alkaloids associated with fescue toxicosis such as ergotamine, ergonovine, and ergovaline affect renal function. The toxicants reduce renal Na⁺/K⁺ ATPase enzyme in rats (Moubarak et al., 2003a). The enzyme plays a major role in optimizing renal filtration and excretion/retention of minerals across the nephron. Serum sodium and potassium levels are decreased and blood urea nitrogen levels are increased in cattle grazing on E+ pasture (Oliver et al., 1997). This is possibly because of α -2 adrenergic receptor activation resulting in decreased antidiuretic hormone release and decreased tubular reabsorption of salt and water (Oliver, 1997). Creatinine, a protein produced by

muscle, is relatively stable in animals and is used to evaluate renal function. Schultze et al. (1999) and Oliver et al. (2000) observed elevated levels of serum creatinine and total bilirubin in cattle grazing E+ pastures and they concluded that these observations were indicative of reduced renal filtration rates.

Alkaline phosphatase is a representative brush border enzyme functionally involved in nutrient absorption and transport of long chain fatty acids in the intestinal mucosa (Kaur et al., 1996). In rats, a majority of serum ALP is intestinal in origin. Alkaline phosphatase activity in rats depends on ADFI status, with a reduction in activity during a 3 to 4 day fast (Holt and Kotler, 1987). Therefore, lowered ALP activity is associated with reduced ADFI, which is the characteristic feature of E+ rats. Reduction in serum ALP activity in E+ fed animals occurs consistently (Rice et al., 1997). Reduction in serum ALP was also observed in steers fed an E+ diet (Schultze et al., 1999).

One of the prominent signs of fescue toxicosis is increased serum ALT levels in E+ treated animals. Alanine aminotransferase, found in hepatocytes and striated muscle cells increases only during hepatocellular injury or necrosis of striated muscles (Valentine et al., 1990). Similar increase in serum ALT levels was observed in steers exposed to fescue toxicosis (Schultze et al., 1999) suggesting liver and skeletal muscle damage.

Blood urea nitrogen (BUN) is an indicator of the amount of urea nitrogen (i.e., a waste product of protein metabolism) and protein metabolism. Blood urea nitrogen levels are reduced in E+ fed animals. Liver damage causes a reduction in blood urea level since urea is synthesized by the liver (Satirapoj et al., 2007). Decreased BUN levels are also seen during malnutrition and impaired nutrient absorption (Preston et al., 1965).

Intake of an E+ diet by rats significantly reduces serum amylase (Spiers et al., 2001); however, steers and heifers on long-term E+ treatment increase serum amylase level (Nutting et al., 1992). Elevated serum amylase is a common indicator of pancreatic toxicity, resulting specifically from damage to the exocrine portion of the gland (Hirano et al., 1992). Bovines lack salivary amylase and therefore exclusively depend on pancreas for the enzyme (Nutting et al., 1992). Although the exact effects of fescue toxicosis on pancreas are not fully understood, it can be speculated that the exocrine portion of the pancreas is affected.

Serum cholesterol is reduced by feeding an E+ diet to cattle (Stuedemann et al., 1985), and rats (Bhusari et al., 2006). It is not known if this reduction is due to increased cholesterol uptake by the tissues or decreased hepatic secretion (Thompson et al., 2001). Cattle grazing on toxic tall fescue during summer months exhibit decreased serum triglyceride levels (Oliver et al., 1995). Gadberry et al. (1995) observed similar results in rats consuming an E+ seed based diet. Stuedemann et al. (1985) made an association between high-nitrogen fertilization of E+ tall fescue, reduced serum cholesterol, and incidence of fat necrosis in cattle. Lowered total serum cholesterol leads to lowered steroidogenesis (Burke et al., 2001) which is another prominent symptom of fescue toxicosis leading to reduced estradiol and progesterone (Burke et al., 2001) concentrations in female cattle. Decrease in the concentrations of estrogen and progesterone may lead to various reproductive problems often seen in E+ animals. Since cholesterol is the precursor for various steroid hormones, lowered cholesterol biosynthesis in E+ animals could be one of the main reasons for the various male and female reproductive problems observed in E+ animals. Although PRL is necessary to

initiate lactogenesis, the involvement of progesterone and estrogen in lactation is significant. Estrogen and progesterone stimulate development of ductal and secretory structures when mammary tissue is primed with insulin, aldosterone, and PRL (Forsyth, 1983). Therefore the common reproductive problems associated with fescue toxicosis could be partly due to the lowered steroidogenesis because of lowered cholesterol biosynthesis.

Oliver et al. (2000) did not observe any changes in serum mineral levels, except copper, in steers grazing on E+ tall fescue. They noted a reduction in serum copper levels in E+ fed steers compared to E- fed steers. Dennis et al. (1998) reported that the reduction in serum copper levels in E+ fed cattle could be because of reduced copper levels in E+ tall fescue.

Insulin-like growth factor is reduced in E+ fed compared to E- fed cattle (Lipham et al., 1989), which they attributed to the reduced feed intake and growth rate. However, there are no consistent effects of E+ tall fescue on serum growth hormone level (Lipham et al., 1989; Oliver et al, 1997). Oliver et al. (2005) hypothesized that growth hormone secretory changes do not contribute to the decreased weight gains associated with E+ tall fescue intake.

Oliver et al. (2000) observed microcytic (smaller in size), hypochromic (less hemoglobin content) erythrocytes in E+ fed compared to E- fed steers, but the number of erythrocytes were greater in E+ compared to E- fed animals. They hypothesized that the reduction in mean corpuscular volume and mean corpuscular hemoglobin is due to the reduced serum copper levels in E+ fed animals which could affect hemoglobin synthesis.

2. 3. 7. 1. *Effects of endophyte-infected fescue on prolactin*

Serum PRL is reduced in livestock (Thompson et al., 1989) and rats (Mizinga et al., 1993) consuming E+ diets. The effect of fescue toxicosis on PRL level is independent of Ta. Aldrich et al. (1993) reported a suppression in PRL level in cattle consuming E+ during exposed to heat (32°C) and thermoneutral conditions (22°C). Lambs fed on E+ (10%) diets had a 93.6% reduction in serum PRL levels compared to lambs fed the E- diet (Gadberry et al., 2003). They reported that changes in PRL are more sensitive to fescue toxicosis than feed intake, skin temperature and Ta. Pure ergovaline reduced PRL concentrations when injected into cattle (McCullough et al., 1994), added to pituitary culture system (Strickland et al., 1994) and when added to diet in lambs (Gadberry et al., 2003).

Ergot alkaloids are potent dopaminergic agonists and pituitary secretion of PRL is inhibited by dopamine stimulation (Lamberts and Macleod, 1990). The central nervous system and pituitary have an abundance of dopamine receptors (Thompson et al., 2001). Depressed serum PRL in animals consuming E+ diets indicates an impact of ergot alkaloids on both pituitary and neural function. Mizinga et al. (1993) studied the effects of endophyte on hypothalamic and striatal dopamine D2 receptor density and binding affinity in rats fed E+ seed (EV concentration was 1.91 micrograms/g) for 21 days. They observed that endophyte did not change the dopamine D2 receptor density, but increased D2 receptor affinity for ergot alkaloids.

Dull and shaggy hair coat changes associated with fescue toxicosis may also be a result of depressed serum PRL (Thompson et al., 2001). Decreased serum prolactin levels in the Djungarian hamsters are an important component of the change into winter pelage

(growth of a whitish winter coat) (Niklowitz and Hoffmann, 1988). Lipham et al. (1989) treated E+ fed steers with metaclopramide (dopamine antagonist) and observed increased serum PRL, improved hair coat quality, and BW gain. From these results, it can be speculated that retention of rough hair coat (Aiken et al., 2006) often seen in cattle on E+ pastures could partially be associated with hypoprolactinemia.

Prolactin receptors are found not only in mammary glands but also in liver, kidney, cerebral cortex, and seminal vesicles (Turington et al., 1972). Decreased PRL levels lead to agalactia, lowered androgen dependent enzyme activity (Prasad et al., 1989), impaired spermatogenesis (Jabbour et al., 1998), lowered serum testosterone levels (Regisford et al., 1993), and immune suppression (Reber et al., 1993) in large animals. Prolactin plays an important role in regulation of hepatic lipogenesis rate, insulin receptor number, ADFI, immunomodulation, water electrolyte balance, and temperature regulation (Strickland et al., 1993). Other effects of PRL in mammals include synergistic effects with steroid hormones on gonad functions and effects on male accessory glands; in addition distribution of PRL receptors in various tissues such as liver, kidney, cerebral cortex, seminal vesicle suggests the involvement of PRL in various physiological processes (Strickland et al., 1993). Therefore hypoprolactinemia associated with fescue toxicosis could affect many vital organ functions in the body.

2. 3. 8. Effects of endophyte-infected fescue on immune system

Steers grazing on E+ tall fescue are found to have lowered immune function that is associated with copper deficiency (Saker et al., 1998). Filipov et al. (1999) challenged the steers, fed an E+ diet for eight months, with acute lipopolysaccharide (LPS). They observed a higher immune-inflammatory response in the E+ group as reflected by higher

values of serum cortisol and tumor necrosis factor- α , and suggested this is an indication of a sensitized hypothalamic-pituitary-adrenal axis and immune system. Likewise, in a separate study, they observed an increase in the levels of proinflammatory cytokine IL-6 in ergotamine treated mice (Filipov et al., 1999). Cattle feeding on E+ diet have suppressed phagocytic activity, as evidenced by reduction in monocyte major histocompatibility complex class II (MHC-II) (Saker et al., 1998). Schultze et al. (1999) and Oliver et al. (2000) reported a reduction in total eosinophil count, γ -globulin levels in cattle chronically fed an E+ diet. Furthermore, Oliver (1997) reported that chronic ingestion of E+ diet by cattle increased thrombogenic (i.e., clot formation) activity and acute phase proteins (i.e., von Willebrand factor) levels, which serve as biomarkers of endothelial cell damage (Hirano et al., 2000). Oliver et al. (2000) observed the elevation of thromboxane levels and platelet aggregation in E+ fed steers. Effects of ergot alkaloids on the immune system add further to the complexity of fescue toxicosis.

2. 3. 9. Effects of endophyte-infected fescue on endothelial cells

Clinical signs associated with fescue toxicosis are due, in part, to effects of alkaloids on vascular endothelium, with resultant effects leading to thickened blood vessels, increased vascular tone, hypercoagulability, ischemia, and gangrenous necrosis of tissues (Oliver, 2005). Thompson et al. (1950) identified vascular endothelium as the cellular target for initial injury by ergot alkaloids. Schultze and Oliver (1999) exposed bovine endothelial cells to ergot alkaloids (ergovaline and ergine) at a concentration of 10^{-5} M. Ergine, at this dose level, only caused minimal effects, however ergovaline caused marked cytotoxic effects. In a separate study, ergovaline was cytotoxic to endothelial cells at a concentration of 10^{-4} M, while ergine had only a slightly toxic

effect. Therefore, Oliver (2005) hypothesized that ergot alkaloid-induced toxicity on endothelial cells *in vivo* results in elaboration of inflammatory mediators and also changes the clotting factor profile. A case study in humans (Pietrogrande et al., 1995) reported the damage of endothelial tissue and platelet dysfunction due to the chronic use of ergotamine in treatment of migraine headaches. Strickland et al. (1996) tested the effects of various ergot alkaloids (ergonovine, α -ergocryptine, ergovaline, N-acetyl loline) on isolated bovine vascular smooth muscle cells from the dorsal metatarsal artery. They observed mitogenic effects of these alkaloids on E+ fed bovine blood vessels leading to well-described thickening of blood vessels. The blood vessel thickening in E+ tall fescue-fed cattle was also reported earlier (Garner and Cornell, 1978; Oliver and Schultze, 1997). Oliver and Schultze (1997) reported that the ergot alkaloids damage vascular endothelial cells, allowing ergot alkaloids to come in contact with various mitogenic agents in the underlying smooth muscle cell layers, resulting in vascular smooth cell proliferation. Reduced lumen size resulting from mitogenesis might contribute to the vascular complications that arise in E+ fed animals (Aldrich-Markham et al., 2003). Oliver (2005) reported inflammation as an important etiological factor for various pathological changes that occur in E+ fed animals. He linked various clinical signs associated with fescue toxicosis, such as febrile response, hyperanalgesia, lameness, and blood stasis in peripheral vessels that leads to coagulation defects, to inflammation. Ergot alkaloids also affect biologically active substances released by endothelial cells, such as increased angiotensin converting enzyme, which leads to increased angiotensin II levels in the sera of E+ fed cattle. Angiotensin II is a potent vasoconstrictor that impacts fluid and electrolyte balance through antidiuretic hormone and aldosterones release

(Oliver, 2005). Cattle exposed to E+ diet are reported to have greater levels of von Willebrand factor (Vwf), which increases the risk for hypercoagulability and thrombosis (Oliver, 2005). Oliver et al. (1998) reported increased levels of thromboxane A₂ (TXA₂) in cattle that graze E+ fescue. Thromboxane A₂ is a potent vasoconstrictor that is formed in blood platelets, and its increased level suggest thrombotic events (vascular occlusions) occurring in E+ fed cattle (Oliver, 2005). Oliver et al. (2000) found reduced serum arginine (a precursor of NO) level in E+ fed cattle, resulting in nitric oxide (NO) deficiency, which contributes to increased vasoactivity and blood platelet activation. They also observed a significant reduction in total nitrite and nitrate levels in E+ fed cattle, supporting the possibility of arginine-deficient status of E+ cattle. However, they did not observe any difference in NO synthase activity between control and E+ fed cattle. Nitric oxide synthase enzyme converts arginine to citrulline and it frees NO for vasodilation. Al-Tamimi et al. (2007) reported a relief in E+ fed rats from HS, when they were treated with NO donor agents. They also demonstrated that treatment with NO donor agents overrides peripheral vasoconstrictive effects of E+ diet. In another study, they compared ergovaline-treated cattle with controls during HS. They observed that nitroglycerine could reverse the impact of HS in ergovaline-treated cattle, but not in control group. Similar results were observed in ergot intoxicated humans, suffering with vasospasm, when NO donor agents (nitroglycerine, nitroprusside) were used (Ashenburg and Phillips, 1989). This suggests that vasodilators such as nitric oxide allow the animal to reduce heat load under HS and thereby alleviate some of the clinical signs associated with fescue toxicosis.

2. 4. Economic impact associated with endophyte-infected fescue

Fescue toxicosis is one of the most notorious grass-related livestock problems in the United States, affecting more than 8.5 million beef cows and 700,000 horses (Stuedemann and Seman, 2005). Annual economic losses to the beef industry from decreased conception rate and weaning weight associated with fescue toxicosis have been estimated at greater than \$600 million (Hoveland, 1993). Further economic impacts occur for the horse and sheep industry (Thompson et al., 2001). In addition, altered thermoregulatory ability, decreased feed intake and reduced average daily gain in older animals' further increase the adverse economic effect of fescue toxicosis (Spiers et al., 2005a). Considering inflation and losses to other livestock (e.g., dairy cattle, horses and sheep), the economic cost associates with this problem may be close to \$1 billion per year (Panaccione et al., 2001).

2. 5. Hepatic detoxification

Xenobiotic metabolizing enzymes play a central role in metabolism, elimination, and detoxification of drugs and foreign compounds introduced into the body (Xu and Kong, 2006). The detoxification system in animals is extensive, highly complex, and influenced by myriad of regulatory mechanisms. The liver plays a major role in toxicant clearance by biotransforming xenobiotics (Phase I metabolism) and the subsequent conjugation (Phase II) of the resulting metabolites into compounds that can be excreted from the body (Phase III) (Mannering, 1985). Most of the tissues and organs in the body are well equipped with various xenobiotic metabolizing enzymes, including cytochrome P450 (CYP), antioxidants, and drug efflux proteins (Xu and Kong, 2006). These enzymes are present in abundance either at the basal, unstimulated level and/or are inducible to an

elevated level after exposure to xenobiotics (Xu and Kong, 2006). Cytochrome P450 enzymes are associated with several biological interactions involving hydroxylation, epoxydation, oxygen and nitrogen dealkylation, oxidative deamination, dehydrogenation, etc. (Bhaskarannair et al., 2006). While some P450 reactions are essential for the removal of xenobiotics from the body, these reactions are also involved in the activation of xenobiotics to active metabolites (Bhaskarannair et al., 2006). All aerobic cells contain a battery of defenses, in the form of antioxidants, against reactive oxygen species and active metabolites formed by CYPs (Tiedge et al., 1998). Cells containing low levels of antioxidant enzymes are more vulnerable to oxidative damage (Tiedge et al., 1998). The ATP-dependent drug efflux proteins are responsible for active transport of xenobiotics and cellular metabolites out of the cell (Kalitsky-Szirtes et al., 2004). Phase I, II and III enzymes act in great co-ordination to detoxify various toxicants or/and chemicals in an animal.

2. 5. 1. *Cytochrome P450*

Cytochrome P450 (CYP) is a generic term for a large number of evolutionary-related oxidative enzymes that are important in animal, plant, and bacterial physiology. Homologs for CYPs have been sequenced from all lineages of organisms, including mammals, birds, fish, insects, worms, sea squirts, sea urchins, plants, fungi, slime molds, bacteria, and archaea (Zuber et al., 2002). Animal CYPs are primarily membrane-associated proteins, located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. The CYPs metabolize thousands of endogenous and exogenous compounds. Interestingly, most CYPs can metabolize multiple substrates, and many can catalyze multiple reactions, which accounts for their central importance in

metabolizing many endogenous and exogenous molecules. Cytochrome P450 isoforms are predominantly present in liver and metabolize various drugs and toxic compounds, as well as metabolic by-products. Cytochrome P450 enzymes are also present in many other tissues of the body, including the mucosa of the gastrointestinal tract, and plays important roles in hormone synthesis and breakdown (including estrogen and testosterone synthesis and metabolism), cholesterol synthesis, and vitamin D metabolism.

The major CYP enzymes involved in metabolism of drugs or exogenous toxins are the CYP3A4 (orthologous to rat CYP3A1), 1A1, 1A2, 2D6 and 2C enzymes (Zuber et al., 2002). The amount of each of these enzymes present in the liver reflects their importance in drug metabolism. Rat CYP1A2 is one of the first isolated and characterized enzymes and is an important drug-metabolizing enzyme in liver (Bertilsson et al., 1994). Cytochrome P450 1A1 along with UDP-glucuronosyl-transferase plays a crucial role in the metabolism of xenobiotic compounds (Nishizawa et al., 2005). The CYP3A4, along with its closely related isoforms CYP3A5, are responsible for majority of drug metabolic pathways, and they are also the most abundant form (30 to 60% of total CYP isoforms in liver) in hepatic tissue. Cytochrome P450 3A7 has a specific role in 16 α -hydroxylation of steroids (Daly, 2006), and bioactivation of several pro-mutagens, including aflatoxin B1 (Li et al., 1997). The CYP2C enzymes are one of the most abundant CYP isoforms in rat liver and they are associated with oxidation of a fungal toxicant, aflatoxin B1, along with dihydropyridines. They also hydroxylate various steroids (Zuber et al., 2002). Their expression and activity of various CYPs depend on multiple factors such as food, genetics, environment, etc (Bauer et al., 2004).

Cytochrome P450 2E1 induces chronic oxidative stress by generating more reactive oxygen species which further lead to hepatocyte injury and cell death (Schattenberg et al., 2004). Hepatocytes containing CYP2E1 release diffusible mediators which activate hepatic stellate cells, collagen, α smooth muscle actin, intracellular and secreted collagen type 1 protein, intra and extra cellular H₂O₂ and lipid peroxidation products (Nieto et al., 2002). Therefore, over-expressed CYP2E1 not only perturbs the homeostasis of hepatocytes, but also contribute to hepatic fibrosis, and generate reactive oxygen species (Schattenberg et al., 2004).

Ergot alkaloids produced by various fungal organisms are known to be substrates of CYP3A isoforms. Ergot alkaloid interactions with the CYP3A enzyme system has been previously elucidated (Moubarak et al., 2003b). Moubarak and Rosenkrans (2000) observed *in vitro* hydroxylation of ergotamine by a cytochrome isoform of CYP3A present in beef liver microsomes. In another study, Moubarak et al. (2003b) induced CYP3A levels in rat liver microsomes and then treated the preparation with ergotamine. This produced a greater metabolism of ergotamine than in controls. Peyronneau et al. (1994) reported metabolism of bromocriptine, an ergot alkaloid similar to ergotamine, by CYP3A in rat liver microsomes treated with dexamethasone, a CYP3A inducer. They also reported that ergopeptides, like bromocriptine, ergocryptine and dihydro-ergotamine, exhibit greater affinity for rat CYP3A. Althus et al. (2000) observed the metabolism of another ergot alkaloid, α -dihydroergocryptine, by CYP3A isoforms. Zanzalari et al. (1989) and Thompson et al. (2001) reported an increase in hepatic mixed function oxidases in sheep consuming E+ diet. These results suggest that ergot alkaloids are

metabolized by various CYP isoforms; however it is not clear if the metabolites are less toxic than the parent compounds.

Transcriptional activation of CYPs is under the control of various nuclear receptors. Nuclear receptors, pregnane X receptor (PXR) and retinoid X receptor (RXR), are ligands that are activated by a large number of structurally and pharmacologically diverse endogenous (steroid hormones, vitamins E and K, bile acids) and exogenous compounds (rifampin and protease inhibitors), and environmental contaminants (organochlorine pesticides) (Lamba et al., 2004). Pregnane X receptor serves as a generalized sensor of hydrophobic toxins (Kliewer et al., 2002). Its activation results in the induction of an elaborate network of CYP isoforms involved in all aspects of xenobiotic detoxification and excretion (Kliewer, 2003). It is mainly expressed in liver, small intestine, and colon (Bertilsson et al., 1998). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor and binds to an upstream element in CYP genes (Waxman, 1999). This is followed by recruitment of coactivator protein, SRC-1 (steroid receptor coactivator-1), for transcriptional activation of the CYP gene (Savas et al., 1999). Given the fact that CYP isoform expression is affected by various factors, there are other nuclear receptors, such as aromatic hydrocarbon receptors, peroxisome proliferator-activated receptor- α , and the constitutive androstane receptor, which induce various CYP isoforms.

2. 5. 2. Antioxidants

As a consequence of increased CYP-associated xenobiotic metabolism, reactive molecules, which may be more toxic than the parent molecule, are produced (Liska, 1998). Another potentially damaging effect of Phase I activity is the production of

oxygen-free radicals that occurs as a result of CYP activity (Percival, 1997). If these reactive molecules are not further metabolized by Phase II conjugation, they may cause damage to proteins, RNA and DNA within the cells (Percival, 1997; Liska, 1998).

Several studies have shown the evidence of association between induced Phase I and/or decreased Phase II activities and an increased risk of disease, such as cancer, systemic lupus erythematosus and Parkinson's disease. This association is also implicated in adverse drug responses (Liska, 1998). Phase II conjugation reactions generally follow Phase I activation, resulting in transformation of a xenobiotic into a water-soluble compound (Liska, 1998). The antioxidants can inhibit free radical production by chelating the transition metal catalysts, breaking chain reactions, reducing concentrations of ROS, and scavenging initiating radicals (Scandalios, 1997).

One of the primary enzymatic defenses against reactive oxygen species is superoxide dismutase (SOD), which catalyses the conversion of superoxide anions into H_2O_2 (a powerful and potentially harmful oxidizing agent). Mammalian cells have a SOD enzyme containing manganese at its active site in mitochondria and one with copper and zinc at the active site in the cytosol (Scandalios, 1997). Minor changes in the Mn-SOD may have a significant impact on the antioxidant status of mitochondria; with approximately a 50% reduction in Mn-SOD resulting in a functional decline of oxidative phosphorylation, an increase in oxidative stress, and an increase in rates of apoptosis (Wheeler et al., 2001).

Other phase II enzymes, such as catalase in peroxisomes and glutathione peroxidase (GPx) in cytosol, are involved in the conversion of H_2O_2 to water and molecular oxygen (Liska, 1998). It is anticipated that cells containing low levels of one or

more of these enzymes would be particularly vulnerable to oxidative damage (Liska, 1998; Tiedge et al., 1998). Of the two antioxidant enzymes, catalase is more effective than GPx in protecting against high concentrations of H₂O₂, while GPx gives more efficient protection than catalase when H₂O₂ concentrations are low (Tiedge et al., 1998). The H₂O₂ undergoes hemolytic scission on the inner mitochondrial membrane and mitochondrial matrix causing site-specific damage of critical matrix targets. Its decomposition outside the mitochondria may cause damage to the outer mitochondrial membrane itself and /or cytosolic targets, within the diffusion limit of hydroxyl radical. Furthermore, prolonged exposure to H₂O₂ could lead to lipid peroxidation. Increased cellular H₂O₂ levels reduce SOD activity (Marczuk-Krynicka et al., 2003) resulting in increased superoxide concentrations within the cell, which in turn reduce catalase activity (Marczuk-Krynicka et al., 2003).

Tiedge et al. (1998) reported that cells transfected with SOD and catalase gave better protection against H₂O₂, compared to cells transfected with SOD or catalase alone. Similarly, cells transfected with catalase and GPx are better protection against H₂O₂ than cells transfected with either enzyme alone. This suggests cooperation between antioxidant enzymes in protecting against various oxidizing species.

Lakritz et al. (2002) reported reduced blood glutathione levels in E+ fed cattle under HS environments. Gorini et al. (1988) observed reduction in forebrain glutathione levels in male rats after chronic i.p. treatment with dihydroergocristine and dihydroergocryptine. These results suggest that ergot alkaloids reduce antioxidant potential of the animals thereby making them more susceptible to oxidative stress.

2. 5. 3. *Antiporter system*

Antiporter activity is an important factor in the first pass metabolism of xenobiotics. It is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics (Liska, 1998). Antiporter activity is co-regulated with CYP enzymes, to suggest that it may support and promote detoxification (Liska, 1998). The multidrug resistance gene (MDR 1A and MDR 1B) and multidrug resistance associated protein 2 (MRP 2) are the two genes which encode for antiporter activity in rats (Kalitsky-Szirtes et al., 2003). The MDR proteins are responsible for the active excretion of a wide variety of lipophilic cationic drugs and xenobiotics from liver, kidney and intestine, whereas MDR 2 proteins extrude various lipophilic anions and their glutathione, glucuronide, and sulfate conjugates (Kalitsky-Szirtes et al., 2003). Targeted disruption of the MDR 1a gene in mice increases the concentration of xenobiotic by 2 to 100 fold in brain, liver, GIT (Schuetz et al., 1996). Over expression of genes associated with antiporter system prevent intracellular accumulation of drugs or xenobiotics (Schuetz et al., 1996), suggesting its significance in reducing toxic load within the cell.

2. 6. *Rat as a model for fescue toxicosis*

Development of rat model for fescue toxicosis provides a rapid, economical tool to determine the multiple effects of fescue toxicosis, and allows for preliminary evaluation of potential treatments (Spiers et al., 2005a). Rats exhibit general symptoms that are characteristic of fescue toxicosis. Spiers et al. (2005a) used a dietary daily dose of ergovaline, at a level that is 16 times greater than the amount required to produce similar symptoms in cattle. The reason for the increased dose requirement in rats is that

the metabolic body size is approximately six times greater in rats compared to cattle (Spiers et al., 2005a). As a result of this difference, cattle would be expected to metabolize and eliminate E+ toxicants at a much slower rate compared to rats. In addition, fescue diet may remain in ruminant stomach for a longer period to provide a greater breakdown and extraction of toxicants than in the nonruminants (such as rats). This require a higher dose in rats to produce a response that is identical to that in cattle (Spiers et al., 2005a). Fescue toxicosis sensitive symptoms observed in cattle (reduced feed intake, body weight, activity) are also observed in rats independent of Ta and level of thermal stress (Spiers et al., 2005a). Cattle and rats exhibit similar short-term thermoregulatory responses to E+ tall fescue toxins when tested under comparable thermal conditions representative of similar levels of strain (Spiers et al., 2005a).

2. 7. MICROARRAYS

2. 7. 1. *Introduction*

DNA microarrays are usually used for global gene expression profiling. Recent developments in microarray technology have provided a reliable platform for the study of gene expression, regulation and function (Chaudhuri, 2005). It has tremendous potential in the exploration of disease processes such as cancer, in drug discovery and development, and also in investigations of population genetics. Notable examples of which include, identification of regulatory genes in the cell cycle mechanism, and disease causing genes in malignancies and certain neurodegenerative disorders (Chaudhuri, 2005). Thus, microarray-based studies provided necessary impetus for biomedical experiments, particularly following the human genome sequencing. Traditional methods

in molecular biology work on a ‘one gene in one experiment’ basis, throughput is very limited and the whole picture of cellular function is often difficult to understand. Furthermore, those processes would require a long period before all the functions of a gene and its interactions with other genes could be elucidated. In contrast, DNA microarrays theoretically allow one to monitor the whole genome on a single chip, which makes the technology very appealing as a shortcut towards data analysis, despite the large financial and technical demands. The flexible nature of the microarray technique allows for wide use in all fields of biology ranging from plants to animals and humans, and its potential applications include, but not limited to: “(i) gene expression comparison under different conditions such as in health, disease, after therapeutic interventions and following exposure to drugs and radiation etc. (ii) identifying unique genes related to different sub cellular organelles and tissues, (iii) analyzing gene function in various metabolic pathways (iv) defining gene within an organism and compare it to a reference organism and (v) detecting differences in single nucleotides between genomic samples obtained from similar organisms etc” (Chaudhuri, 2005).

2. 7. 2. Recent applications of DNA microarrays to toxicology

The field of toxicogenomics has progressed rapidly since the application of DNA chips to toxicology was proposed in the late 1990s (Afshari et al., 1999). One of the early concerns about the use of DNA microarray in toxicology was how to properly compare experiments that used a wide variety of commercial and proprietary platforms, protocols, and analysis methods. In US, the National Institute of Environmental Health Sciences (NIEHS) has created the National Center for Toxicogenomics (NCT) to provide a reference system of genome-wide gene expression data and to develop a knowledge base

of chemical effects in biological systems (Tennant, 2002). The NCT has conducted some proof-of principle experiments to establish signature profiles of known toxicants and to link the pattern of altered gene expression to specific parameters of conventional indices of toxicity (Hamadeh et al., 2002). These studies show that it is possible to identify a signature of expressed gene patterns after exposure to a given toxicant (Tennant, 2002). Recently, DNA microarrays have been used to develop a much deeper insight into the mechanism of chemical toxicity at molecular level such as the effects of arsenic, nickel, chromium and cadmium in human bronchial cells (Andrew et al., 2003). This and other toxicogenomics studies show that “(i) patterns of gene expression relating to biological pathways are robust enough to allow insight into mechanisms of toxicity, (ii) gene expression data can provide meaningful information on the physical location of the toxicity, (iii) dose-dependent changes can be observed and (iv) concerns about oversensitivity of the technology are unfounded” (Pennie et al., 2004).

2. 7. 3. Application of microarray technology to fescue toxicosis

Microarray technique has recently been used in the field of fescue toxicosis to identify the affected pathways due to intake of an E+ diet. Bhusari et al. (2006) used mice as a model to study the effects of E+ diet on hepatic gene expression using a rat oligonucleotide array (50 mer) containing 1,353 liver specific probes. They fed the mice with E- or E+ diet for two weeks at TN (24°C) or HS (34°C) conditions. They observed genes associated with sex steroid metabolism, cholesterol and lipid metabolism down-regulated and ribosome and protein synthesis up-regulated in E+ mice under TN conditions. Under HS conditions, genes associated with phase I detoxification, antioxidants, denovo lipogenesis and lipid transport functions are up-regulated and genes

for DNA damage control and unfolded protein responses are down-regulated in E+ mice (Bhusari et al., 2006).

Results from the above mentioned study encourage the use of high throughput microarray technology to understand the global changes in gene expression as a result of an E+ diet intake.

CHAPTER III

SHORT- AND LONG-TERM RESPONSES TO FESCUE TOXICOSIS AT THERMONEUTRALITY OR DURING HEAT STRESS

1. ABSTRACT

Rats were monitored under TN, short-term (SH) or long-term (LH) heat stress (HS) conditions to determine the time-related changes associated with the intake of diets containing either endophyte-infected (E+) or uninfected (E-) fescue seed. Core temperature did not change in rats fed the E+ diet at TN, but increased with SH and LH exposures. The E+ diet reduced feed intake and conversion efficiency, daily gain, serum prolactin and cholesterol during TN and HS exposures. Rats exposed to one stressor (E+ or heat) exhibited greater adaptation than those simultaneously exposed to both stressors, suggesting a greater shift from normalcy with multiple stressors.

2. INTRODUCTION

Tall fescue [*Lolium arundinaceum* (Schreb) Darbysh., formerly *Festuca arundinacea* (Schreb)] is one of the major grasses grown in temperate regions of the world (Zhang et al., 2005). Within the United States, more than 8.5 million cattle graze on tall fescue, and more than half of the tall fescue forage is infected by the endophyte

Neotyphodium coenophialum (Jones et al., 2004). The endophyte maintains a symbiotic relationship with the plant by improving its drought and heat tolerance (Malinowski et al., 2005) but produces toxins (ergopeptine alkaloids) which interact with dopaminergic, adrenergic and serotonergic receptors and reduce the heat tolerance, growth, and performance of cattle grazing on the endophyte-infected tall fescue grass (Cross et al., 1995; Oliver, 2005). The clinical signs associated with consumption of endophyte-infected fescue are collectively referred as fescue toxicosis. Other species, including horses (Cross et al., 1995), sheep (Gadberry et al., 2003), rabbits (Filipov et al., 1998), rats (Spiers et al., 2005a), mice (Varney et al., 1991), meadow voles (Conover, 1998) and birds (Zavos et al., 1993) exhibit some of the clinical manifestations of fescue toxicosis that are seen in cattle.

Like cattle, rats fed a diet containing endophyte-infected fescue seed (E+) exhibit reductions in average daily feed intake (FI) and growth (Spiers et al., 2005a), making the rat a useful animal model for the study of fescue toxicosis under controlled environmental conditions (Osborn et al., 1992; Spiers et al., 2005a). It has been noted that many of the animal responses to ergopeptine alkaloids depend on Ta, with augmented hyperthermic and hypothermic conditions observed in animals fed E+ diets in hot and cold environments, respectively (Neal and Schmidt, 1985; Spiers et al., 1995). Injection of rats with ergovaline (EV), the primary ergopeptine alkaloid found in endophyte-infected fescue, (Zhang et al., 1994) results in hypothermia at TN and hyperthermia under HS conditions.

The combination of altered thermoregulatory ability and reduced caloric intake over an extended period of time will likely have a negative impact on general animal

health and performance. This effect has been documented, in part, using measurements of FI, weight gain, and serum chemical parameters in cattle fed an E+ diet (Oliver et al., 2000). However, little experimental data is available for a detailed comparative evaluation of the effects of ergopeptine alkaloids on these research endpoints under controlled conditions of short- and long-term exposures to heat, and such information would facilitate the characterization of adaptation to one or both of these stressors (*i.e.*, ergopeptine alkaloids and/or heat). The objective of the present study was to evaluate the temporal effects of E+ and/or HS on various physiological responses, including Tc, activity count, FI, feed conversion efficiency (FCE), average daily gain (ADG), selected serum biochemical parameters and visceral organ weights associated with fescue toxicosis. It was hypothesized that there are temporal differences in physiological responses to tall fescue endophytic toxins under thermoneutral, short-term and long-term heat conditions that characterize potentially adaptive processes.

3. MATERIALS AND METHODS

Experimental Design

Three experiments (Figure 3. 1) were performed during which different rats were fed diets containing either E+ or non-endophyte-infected (E-) fescue seed, and maintained under TN conditions (21°C) for five days (pre-heat stress/thermoneutral), or extended under TN or HS conditions (31°C) for either three days (short-term heat stress) or three weeks (long-term heat stress).

Animals and Treatment Diets

Male Sprague-Dawley rats (n = 24/Experiments 1 and 3 and 12/Experiment 2; 42 d of age at start; Charles River Laboratories, Willington, MA), were maintained on a 12 h light/dark cycle, with percent relative humidity ($50 \pm 5\%$) and selected temperature condition as noted in an earlier study (Spiers et al., 2005a). Feed and water were provided *ad libitum* throughout the experiment. Control and treatment diet compositions were previously described, with the treatment diet adjusted to provide a specific amount of EV (Spiers et al., 2005a). Ergovaline content in E- seed was almost negligible (23 ppb), whereas E+ seed contained a concentration of 4,100 ppb on a DM basis as measured by HPLC (detection limit = 50 ppb and CV = 7%; Rottinghaus et al., 1993). The E+ and E- seed were also analyzed for the presence of ergopeptine alkaloids from *Claviceps purpurea* using HPLC (Rottinghaus et al., 1993). All diets were stored at 4°C prior to use. Animal procedures followed guidelines for humane treatment set by the University of Missouri Animal Care and Use Committee.

Treatment Period and Sample Collection

The schedule in all three experiments consisted initially of an eight-day recovery period after intraperitoneal implantation of telemetric transmitters (Model VM-FH; Mini-Mitter Company, Inc., Bend, OR), followed by a five-day pretreatment period (d 1 to 5), during which all rats received E- diet (Spiers et al., 2005a). Intraperitoneal transmitters were used to collect Tc and activity count at hourly intervals, and then averaged to obtain daily values for analysis. Rats in Experiment 1 were randomly assigned to either E- (n = 12) or E+ treatment (n = 12) groups and administered treatment at TN for 5 d (d 1 to 5; Figure 3.1) to determine the fescue toxicosis response in the absence of heat stress.

During short-term heat stress (Experiment 2), rats were exposed to heat stress (n = 6; 2 groups: E- HS and E+ HS) for three days. During long-term heat stress (Experiment 3), rats were divided at random into four groups at the end of the TN period (n = 6; 4 groups: E- TN, E- HS, E+ TN, and E+ HS) to determine adaptive responses to heat stress and fescue toxicosis. Ambient temperature was held at 21°C (TN) during pre-heat period and increased to 31°C (HS) during short- and long-term heat stress periods (Figure 3. 1). At the end of each treatment period, rats from each group were anesthetized using a mixture of ketamine (87 mg/ml) and xylazine (13 mg/ml) administered intramuscularly at 0.1 mg/100 g BW and blood was drawn via cardiac puncture for analysis. Selected visceral organs (i.e., liver, adrenal, kidney, heart, and testes) were collected from each animal, following euthanasia (rat cocktail and exsanguination) and wet organ weights were measured.

Serum Chemistry

Collected blood was centrifuged immediately to obtain the serum, which was frozen (-20°C) for later use. Serum analyses included determinations of blood urea nitrogen (BUN), glucose, ALP, cholesterol and various serum minerals using standard procedures. Most of these measurements are components of a larger biochemical profile produced by the Veterinary Medical Diagnostic Laboratory, University of Missouri-Columbia using an auto-analyzer (Olympus AV400; Olympus America, Inc., Melville, NY). Serum prolactin (PRL) concentrations were determined using rat prolactin enzyme immunoassay kit (SPI – BIO, Massy Cedex, France) as previously reported (Duhau et al., 1991).

Statistical Analysis

Data from the different studies was not combined for TN and short-term HS due to unequal sample sizes. Instead, physiological parameters were only compared between treatment groups within each experiment, and changes from pretreatment Day -1 were determined. Data for Tc and activity count consisted of daily averages, calculated from hourly values within each day, and used for comparison with other daily values (e.g., FI, ADG). Mean for daily values were analyzed as a function of time and treatment group. The above parameters were analyzed by repeated measures analysis of variance (mixed procedure) of SAS (Littell et al., 1998) and standard least square model fit (JMP[®], SAS Institute Inc., Cary, NC). The statistical model consisted of treatment, time, and treatment x time interactions. If a significant difference existed for any comparison, then a Tukey - Kramer multiple comparisons test (Steele and Torrie, 1980) was performed ($\alpha = 0.05$) to determine specific effects. A significant effect implies that the p or α value is less than or equal to 0.05. All data are reported as least square means \pm 1 SE.

4. RESULTS

Pretreatment values at TN

Average daily group Tc ranged from 37.5 to 38.2°C before treatment, with no difference across treatments in all three experiments, and, likewise, average group activity count ranged from 23.2 to 39.1 counts/h and was not different across treatments in each of the three experiments. Pretreatment group averages for FI did not differ

between treatments, and there were no differences between mean group values for body weight as a function of treatment in all experiments. Feed conversion efficiency was calculated as the ratio of changes in body weight to FI multiplied by 100, and there were no differences between group pretreatment FCE values across treatments in any of the experiments

Short- (Experiment 1) and long-term (Experiment 3) responses to treatment diets at TN

Core temperature responses and activity count

The E+ diet produced no significant change in average daily group Tc compared to E- groups during short- or long-term TN studies. Likewise, there was no significant effect of diet on change in average daily activity count by treatment. Changes in daily Tc and activity count over time were absent in both E- and E+ rats during short- or long-term TN conditions.

Feed intake

There was a significant decrease in mean FI of E+ rats on Day 1 that was below the level observed in rats fed E- diet during both short- and long-term periods (Figure 3. 2a). By the end of the short-term experiment (Day 5), E+ rats exhibited signs of adaptation,, with a significant increase in FI from Day 1 level (Figure 3. 2a). Similarly, E+-treated rats during the long-term period reached the mean level of FI for E- rats by Day 9 (Figure 3. 2a).

Feed conversion efficiency (FCE)

Rats fed E+ diet significantly reduced mean FCE compared to E- rats (-38.89 ± 3.05 g BW/g FI versus -10.74 ± 3.05 g BW/g FI) by the end of the short-term experiment.

The initial significant reduction in average FCE of E+ rats occurred on Day 1, with an indication of partial adaptation or recovery by Day 5. Feed conversion for E- rats did not change during this period. Rats on the E+ diet during the long-term experiment also had reduced average FCE (-10.69 ± 1.09 g BW/g FI) compared to E- rats (-6.18 ± 1.09 g BW/g FI). The initial signs of significant reduction in average FCE of E+ rats occurred within the first 24 hours of treatment. This level remained low in E+ rats until Day 3, followed by significant adaptation or recovery by Day 4. The E- rats did not change FCE during this period.

Body weight

Consumption of E+ diet significantly decreased average BW (Figure 3. 2b) below E- level during both short- and long-term periods, and, similarly, the slope for growth rate was significantly reduced for E+ compared to E- rats for both periods (Table 3. 1). The first indication of significant decrease in average BW of E+ rats below E- level occurred on Day 1 (Figure 3. 2b). By the end of the short-term period, average BW of E+ rats was not different from Day 1, and by the end of the long-term period the E+ rats had increased their BW above Day 1 level to suggest partial adaptation or recovery, However, average BW of E+ rats was always below that of rats fed the E- diet on any day.

Serum biochemistry profile

Rats fed E+ diet had mean serum glucose, BUN, cholesterol and ALP values that were significantly below E- level during the short-term period, with no long-term change (Table 3. 2). Endophytic toxins did not alter serum sodium, potassium, calcium, chlorine or phosphorus levels during either period (data not shown).

Visceral weights

Intake of E+ diet consistently resulted in a reduction ($p \leq 0.07$) in mean wet weight of liver, corrected to BW, for short- and long-term periods at TN (Table 3. 3). The E+ group exhibited greater mean weight of testes, corrected to BW, at TN. Mean relative wet weights of heart, adrenal and kidney did not differ among treatment groups for either period (data not presented).

Short- (Experiment 2) and long- (Experiment 3) term responses to fescue toxicosis during HS

Core temperature responses and activity count

Average daily Tc of E- rats increased significantly by 0.22°C from Day 5 at TN to Day 6 of HS. By Day 8, Tc of E- rats exposed to HS had returned to TN level. In contrast, there was a significant increase (0.47°C) in Tc of E+ rats by Day 6, with an additional 0.20°C increase to the end of short-term HS (Day 8).

During long-term HS, average daily Tc of E- rats significantly increased (0.26°C) from Day 5 to 6. Although their Tc continued to increase to Day 7 (0.45°C), by Day 8 it had returned to TN level. The increase in Tc of E+ rats within the first 24 hours of HS (0.42°C) was similar to short-term HS. However, this increase was not significant from Day 5 because the variance was greater for long-term HS. A significant increase occurred on Day 7 from Day 5 for this group with a continued rise in Tc to Day 8, when the increase from Day 5 was 1.26°C . The Tc of E+ rats remained elevated until Day 12 from Day 5 level and by Day 13, values returned to preheat level. There was a significant difference in Tc of E- and E+ groups from Day 8 to 10.

During short-term HS, average daily activity count of E- rats significantly decreased (12.47 counts/h) from Day 5 to Day 8. Mean activity count of E+ rats was significantly decreased (10.07 counts/h) within 24 hours of HS. The E+ rats continued to decrease activity count until Day 8, when the reduction from Day 5 was 15.67 counts/h. There was no significant E+ effect on activity count during short-term HS.

During long-term HS, average daily activity count was significantly decreased (15.31 counts/h; $p < 0.01$) in E- rats by Day 6; however by Day 7, activity count of E- rats returned to pretreatment level. Within 24 hours of HS, activity count of E+ rats significantly decreased (11.19 counts/h). However, by Day 8 the values returned to TN level. The E+ diet did not have a significant effect on activity count of E+ rats during long-term HS.

Feed intake

Heat exposure resulted in a significant reduction in average FI in both E- and E+ rats from Day 5 level during both short- and long-term HS. The first sign of significant reduction in FI occurred within 24 h of HS. During short-term HS, FI of E- rats was still below Day 5 level on Day 8, however, E+ rats had returned to Day 5 level. This was likely because FI for E+ rats was already extremely depressed on Day 5 and there was little room for change. During long-term HS, FI values of E- and E+ rats never returned to Day 5 level, even by the end of Day 26. Feed intake values were nearly always significantly lower in E+ fed rats compared to E- rats during this period (Figure 3a).

Feed conversion efficiency

Heat exposure during short-term HS significantly reduced average FCE on Day 6 in both E- (101.44 g BW/g FI) and E+ (80.66 g BW/g FI) rats from Day 5 level.

However, complete adaptation occurred in both treatment groups by Day 8, with return to TN level. There was no treatment effect on FCE during short-term HS. During long-term HS, FCE of E- (135.37 g BW/g FI) and E+ (202.95 g BW/g FI) groups on Day 6 were significantly decreased from Day 5 level. Complete adaptation and return to TN level occurred on Day 8 for E- rats and Day 9 for E+ rats. Treatment effect on FCE was also absent during long-term HS.

Body weight and growth rate

During short-term HS, initial signs of significant reduction in mean BW from preheat level occurred in E+ rats on Day 7, whereas E- rats did not exhibit this reduction. Body weight and growth rate slopes (Table 3. 5) were lower in the E+ compared to the E- groups during short-term HS (Figure 3. 3b). During long-term HS, there was an initial reduction in BW on Days 6 and 7 below preheat level for E+ and E- rats, respectively. Body weight of E- rats exhibited signs of adaptation to HS by Day 8, with a steady increase in growth rate after this time. Reduction in BW of E+ rats continued until Day 8, and by Day 9 there were signs of adaptation. Similarly, growth rate of E+ rats exhibited partial adaptation to fescue toxicosis under HS and the values were not significantly different from their E- counterparts (Table 3. 1).

Serum biochemistry profile

Short-term HS reduced serum glucose level in both E- and E+ rats compared to TN counterparts; however, such an effect was absent during long-term HS (Tables 3. 2 and 3. 4). Long-term, but not short-term HS, decreased serum cholesterol and ALP levels in E+ compared to E- rats (Table 3. 3). Both HS exposures increased serum PRL above TN level in E- and E+ rats (Tables 3. 2 and 3. 4; no statistical comparison). Serum PRL

levels were significantly reduced in E+ compared to E- rats during both experiments (Table 3. 3). Endophytic toxins did not alter serum sodium, potassium, calcium, chlorine or phosphorus levels during short- and long-term HS (data not shown).

Visceral weights

The E+ diet significantly reduced BW-specific wet liver weight during all exposures (Table 3. 3). Long-term HS reduced BW-specific wet liver weight in E- and E+ rats compared to TN conditions (Table 3. 3). Such a comparison could not be performed for short-term exposure since it encompassed two different experiments. Body weight-specific wet testis weight was greater in E+ rats only during long-term HS ($p < 0.01$), but not during the short-term HS ($p \geq 0.10$; Table 3. 3). Long-term HS increased BW-specific wet testis weight only in E+ rats compared to long-term TN (Table 3. 3). In contrast, there was no effect of any treatment on BW-specific wet weights of heart, adrenal and kidneys (data not presented).

5. DISCUSSION

Core temperature and activity count

There was no significant change in daily Tc of E+ rats at TN in the present study. Others have observed a prominent acute reduction in rectal temperature of rats following ip injection of EV at TN (Zhang et al., 1994; Spiers et al., 1995). Likewise, there is a reduction in Tc of rats during intake of an E+ diet at TN (Spiers et al., 2005a). However, this reduction, albeit significant, was only 0.2°C and well within the daily circadian cycle for normal Tc. In a more recent study, Settivari et al. (2006) noted that night Tc was

significantly reduced within several days on an E+ diet at TN. However, this response was transient and was not evident at the end of TN exposure. In support to the present findings, Osborn et al. (1992) observed no change in rectal temperature of steers fed an E+ (seed) diet for 28 days under TN conditions. Inconsistencies observed with regard to the effects of fescue toxicants on Tc at TN warrant additional studies.

Short- and long-term exposure to HS increased average daily Tc of E- rats in the present study. This Tc response is corroborated by previous reports noting that exposure of male rats to 32 to 33°C for 10 to 14 days (Shido et al., 1989; Matthew, 1997) results in elevation of average Tc above TN level. Adaptation of average daily Tc to HS in the present study is also in agreement with previous reports (Matthew, 1997). Even with the elevated mean core body temperatures in rats fed E- diet during HS, E+ fed rats in short- or long-term HS consistently exhibited increased average daily Tc compared to E- fed rats, which is in agreement with the reports of Spiers et al. (2005a). Fescue toxicosis produces a similar increase in body temperature in sheep (Hemken et al., 1979) and steers (Osborn et al., 1992) during heat stress. This increase in temperature is attributed to increased peripheral vasoconstriction (Aldrich et al. 1993), as a result of a reduction in NO level (Al-Tamimi et al., 2007). Dietary long-term treatment of rats fed an E+ diet with an NO donor (molsidomine; 120 mg/ml drinking water) during heat stress (32°C) reduces night hyperthermia associated with fescue toxicosis by ~0.4°C (Al-Tamimi et al., 2007). Adaptation observed in average daily Tc of E+ rats to HS are in agreement with the observations of Spiers et al. (2005a). Rats adapt to HS by decreasing metabolic heat production (Gordon, 1993), and increasing heat loss activities (Horowitz et al., 1983) and

this might explain the recovery observed in Tc values of E+ and E- fed rats. Additional studies are needed to verify this.

Intake of an E+ diet under TN conditions in the present study did not produce a consistent change in average daily activity count compared to E- fed rats. Spiers et al. (2005a) observed a slight decrease in activity count of E+ compared to E- rats during TN, which they attributed to added effects of stress and general sickness behavior.

In the present study, short- and long-term HS reduced the general activity count of E- rats. Al-Tamimi et al. (2007) observed 32 to 34% decrease in average activity count in rats exposed to HS conditions (32°C), with greater reduction occurring during night than day hours. They speculated that rats reduce activity count during HS to minimize movements and thereby heat production, for reduction of heat load. Similar response of rats to heat exposure has been reported by others (Spiers et al., 2005a; Gordon, 1993). However, Spiers et al. (2005a) did not observe changes in activity count of E- rats during long-term heat stress. As for Tc, this response appears to be transient and not consistent across different studies. Intake of an E+ diet during HS did not alter activity count of E+ compared to E- fed rats in the present study, although Spiers et al. (2005a) reported a long-term reduction in activity count in E+ compared to E- fed rats under this condition. Additional studies are needed to determine the definitive effects of E+ diet on activity count during heat exposure.

Feed intake

The effect of endophytic toxins on FI have been extensively studied in both ruminants and monogastrics, and include rats (Spiers et al., 2005a; Roberts et al., 2002) and steers (Osborn et al., 1992) under TN and HS conditions. The E+ diet in the present

study produced a decrease in mean FI within the first 24 hours at TN, demonstrating that this particular response is independent of *Ta*. Heat stress-induced reduction in mean FI of E- rats, in the present study (Figure 3. 3a) is in agreement with earlier reports (Johnson and Cabanac, 1982; Spiers et al., 2005a). Al-Tamimi et al. (2007) reported that such reduction in FI is a means for the heat-stressed animals to reduce their postprandial heat increment and is a requirement to dissipate excess heat. Although, mean FI returned to TN level during short-term HS, this adaptation did not occur during long-term HS. Spiers et al. (2005a) did not observe signs of adaptation in FI of E- rats under HS conditions. endophytic toxins. Reasons for the reduced caloric intake in E+ treated animals have not been determined. Spiers et al. (2005a) ruled out the likelihood of palatability of ground seed or amount of seed fed as the possible causes for the observed reduction in caloric intake, since the E- diet containing similar content of fescue seed did not alter FI. Partial FI recovery observed in E+ rats is in agreement with earlier reports (Spiers et al., 2005a).

Feed conversion efficiency

Consumption of an E+ diet at TN decreased FCE of E+ compared to E- rats. Exposure to heat stress reduced FCE of both E- and E+ rats in the present study and has previously been reported to reduce FCE (Baccari et al., 1983; Suk et al., 1995; Washburn et al., 1995; Cooper and Washburn, 1998). During HS, E+ intake reduced FCE in E+ compared to E- rats, however, the difference was statistically insignificant due to greater variance. Although FCE and FI recovered in E+ rats during TN and HS, BW did not exhibit similar recovery. Reduced FCE in E+ rats could hinder the ability to utilize and convert feed energy into productive aspects, and in farm animals could contribute to impaired production associated with fescue toxicosis. Reduced FCE was reported earlier

in E+ fed rats (Neal and Schmidt, 1985) as well as in steers (Schmidt et al., 1982; Hoveland, 1993). Donaldson et al. (1991) and Osborn et al. (1992) noted that reduced growth rate in E+ steers, although maintained on high nutrient feed, was likely due to reduced FCE. Al-Tamimi et al. (2007) observed 88.5% depression in FCE of E+ rats under similar HS conditions from FCE values during HS alone. The toxic effects of ergovaline at the level of gastrointestinal tract epithelium partially explain the suppressed FCE in E+ rats.

Average daily gain

Poor weight gain is considered as one of the classical signs of fescue toxicosis. In the present study, intake of an E+ diet under TN conditions reduced mean ADG and growth rate in short-term experiment. However, during chronic exposure (Experiment 3), only a partial adaptation occurred for growth rates of E+ rats, despite complete recovery of FI and FCE. Both short- and long-term HS decreased ADG and growth rate of E- rats to comparable level. The E+ diet intake under short-term HS decreased growth rate to minimal level, however E+ rats during long-term HS exhibited partial adaptation with a growth rate that was similar to E- rats. Reduced growth rate under HS conditions can once again be attributed to reduced FI and FCE. Similar decrease in ADG have been observed in rats (Spiers et al., 2005a; Al-Tamimi et al., 2007), sheep (Gadberry et al., 2003), and cattle (Osborn et al., 1992) fed an E+ diets under TN or HS conditions. Ergopeptine alkaloids are reported to affect hepatic mitochondrial genes associated with ATP production at TN (Settivari et al., 2006), which might explain for reduced growth rate in E+ rats, in spite of the recovery observed in FI and FCE.

Serum parameters

Reduction in serum PRL level is considered to be a reliable verification of fescue toxicosis (Schultze et al., 1999). The hypoprolactinemia observed in the present study is most likely due, at least in part, to the inhibitory effects of ergopeptine alkaloids on lactotrophic cells present in the anterior pituitary (Strickland et al., 1993). Intake of the E+ diet in the present study reduced mean serum PRL concentrations under both TN and HS conditions when compared to E- rats. Variations observed in E- and E+ rat serum PRL levels during the three studies could be due to seasonal variation in PRL secretion in laboratory rats (Vazquez et al., 2006). Rats in the present study were tested between June and November. Prolactin increases the circadian rhythm of lipogenesis and insulin receptor numbers in hepatic tissue, and inhibition of PRL secretion decreases hepatic lipogenic rates (Cincotta and Meier, 1989). Furthermore, PRL stimulates rat FI and BW in a dose-dependent manner (Gerardo-Gettens et al., 1989). Therefore, hypoprolactinemia associated with fescue toxicosis could contribute to reduced daily gain in E+ rats. In addition distribution of PRL receptors in various tissues such as liver, kidney, cerebral cortex, seminal vesicle suggests the involvement of PRL in various physiological processes (Strickland et al., 1993); and therefore hypoprolactinemia associated with fescue toxicosis could affect many vital organ functions in the body.

Exposure to HS, increased serum PRL level compared to TN exposure. Previous studies have shown that HS increases circulating PRL level in rats (Neill, 1970), cows (Johke, 1970) and poultry (Johnson, 1981).

Intake of an E+ diet decreased serum cholesterol level in E+ fed rats in both environments, with the exception of short-term HS when E+ rats exhibited only a tendency towards reduction. Stuedemann et al. (1985) observed a similar reduction in serum cholesterol levels of cattle grazing on E+ fescue. Oliver et al. (2000) observed reduced serum cholesterol levels in steers grazed on endophyte-infected tall fescue for three years. Reduction in serum cholesterol levels is because ergopeptine alkaloids down-regulate various genes associated with cholesterol biosynthesis (Settivari et al., 2006; Bhusari et al., 2006). Lowered total serum cholesterol leads to lowered steroidogenesis (Burke et al., 2001), which contributes to various reproductive problems often seen in E+ animals. Heat stress in the present study, did not affect serum cholesterol levels. However, earlier studies reported negative effects of HS on serum cholesterol levels in cows (Fuquay et al., 1980).

In rats, most ALP is located in the brush border of intestinal cells and is functionally involved in nutrient absorption and transport of long chain fatty acids in the intestinal mucosa (Kaur et al., 1996). Serum ALP concentrations in rats depend on FI status, with a reduction occurring in fasting rats (Holt and Kotler, 1987). Endophytic toxins reduced serum ALP during both short-term TN and long-term HS in the present study. Therefore, reduction in serum ALP in E+ rats might represent reduced caloric intake. Reduction in serum ALP was earlier reported in steers fed E+ diet under HS conditions (Schultze et al., 1999). Reduced serum glucose levels in E+ rats further represent reduced caloric intake leading reduced growth rate in E+ rats. Heat stress in the present study increased serum ALP in E+ rats during short-term exposure, but decreased in E- and E+ rats during long-term HS compared to E- and E+ rats at TN. Interestingly,

these results differ from those of Harada et al. (2003), who, observed that severe heat stress (43°C for one hour) induces gene and protein expression of intestinal alkaline phosphatase in rats. The difference may be the intensity and duration of heat exposure.

Heat stress exposure in the present study reduced serum glucose levels in both E- and E+ rats during short-term HS compared to short-term TN conditions, with no changes during long-term exposures. Heat stress is known to increase glucose transport in the intestine to compensate for reduced caloric intake (Garriga et al., 2005), resulting in reduced serum glucose levels. Blood urea nitrogen level, a blood indicator of protein metabolism, was also reduced during impaired nutrient absorption (Preston et al., 1965). Endophytic toxins reduced BUN concentration in the present study only during short term TN, with no difference between the treatment groups during long term TN and HS periods. Heat stress did not affect BUN levels in E- and E+ rats in the present study. However, Lee et al. (2005) reported increased levels of BUN in heat-stressed rats.

Visceral organ weights

Intake of E+ diet reduced average liver weight corrected to BW in both TN and HS, agreeing with an earlier report by Chestnut et al. (1992). This reduction in liver weight could be due to the combined effects of E+ toxicants and reduced caloric intake. Exposure to short- and long-term HS reduced average liver weights corrected to BW. These results can be partially attributed to the additional reduction in FI and ADG of HS rats compared to rats maintained at TN (Gursoy et al., 2001). Testis weight relative to body weight was greater in E+ compared to E- rats during TN and HS. However, this increase in relative weight could be because of a greater reduction in BW than testis weight.

Adaptive responses

In the present study, rats fed either E- or E+ diets exhibited signs of adaptation to HS, with respect to T_c , after initial peaks during HS. However, E- rats recovered better than rats fed E+ diets and exposed to similar HS. These findings are consistent with the observations of Horowitz and Meiri (1993) and Matthew (1997), who exposed rats to 34 or 32 to 33°C for 14 days and observed that T_c declines 2 to 3 days after HS. Although not measured in the present study, reductions in metabolic heat production or increases in heat loss mechanisms are two ways rats adapt to HS. Decreased metabolic heat production during summer is achieved through reduced caloric intake and thermogenic thyroid hormone production (Baccari et al., 1983). Similarly, rats increase their heat loss mechanisms during heat stress by increasing splanchnic and peripheral vasodilatation (Horowitz, 1998), respiratory (80%) and salivary (85%) evaporative water loss (Schmidek et al., 1983), cutaneous evaporative water loss from skin surface by licking highly vascularized body surfaces (paws, scrotum, tail), spreading saliva over the body surface (Hubbard et al., 1982), and increasing the effective surface area for heat dissipation (through extended body parts) (Gordon, 1993). Activation of genes associated with gluconeogenesis, lipid metabolism and protein catabolism and hepatic detoxification mechanisms (e.g., cytochrome P450 isozymes) in response to E+ diet intake (Settivari et al., 2006) may partially contribute to the adaptation observed in E+ rats.

6. CONCLUSION

Changes that occur during fescue toxicosis not only involve short-term and long-term pathologies associated with distress, but also recovery that occurs over time. In fact, the recovery observed in chronic exposure may be more representative of the problems associated with fescue toxicosis than the acute effect. Rats fed ergopeptine alkaloids at thermoneutrality adapted to fescue toxicosis, as reflected by the complete recovery in feed intake and partial recovery in weight gain. However, rats simultaneously exposed to a second stressor (i.e., heat), failed to show a similar rate of recovery. The long-term depressant effect of heat stress seems to have a greater influence under these conditions than intake of the endophyte diet which seemed to have a more transient response.

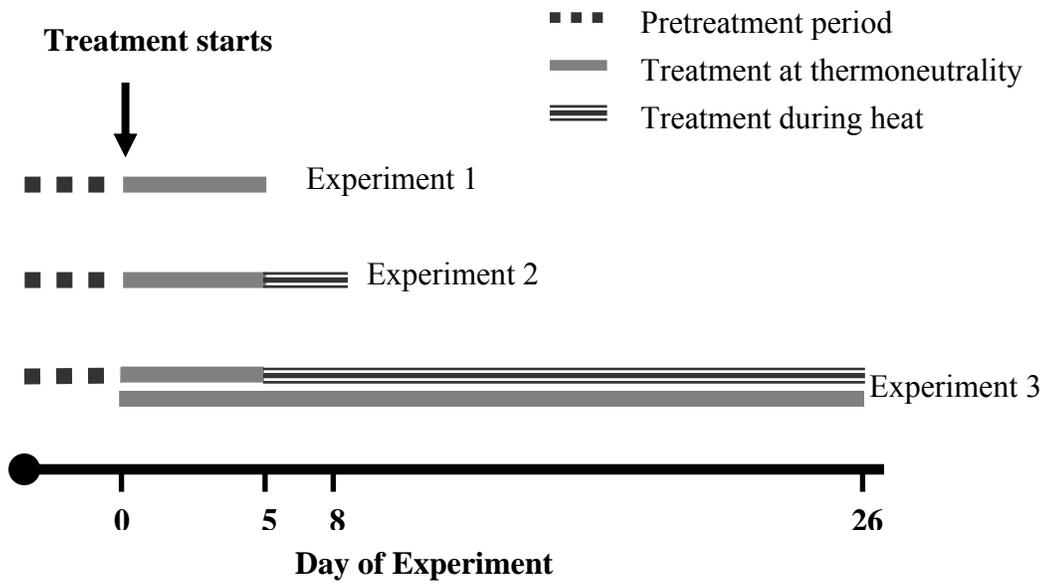


Figure 3. 1. Study design for the Experiments. Ambient temperature was maintained at 21°C during thermoneutral exposure and at 31°C during heat exposure.

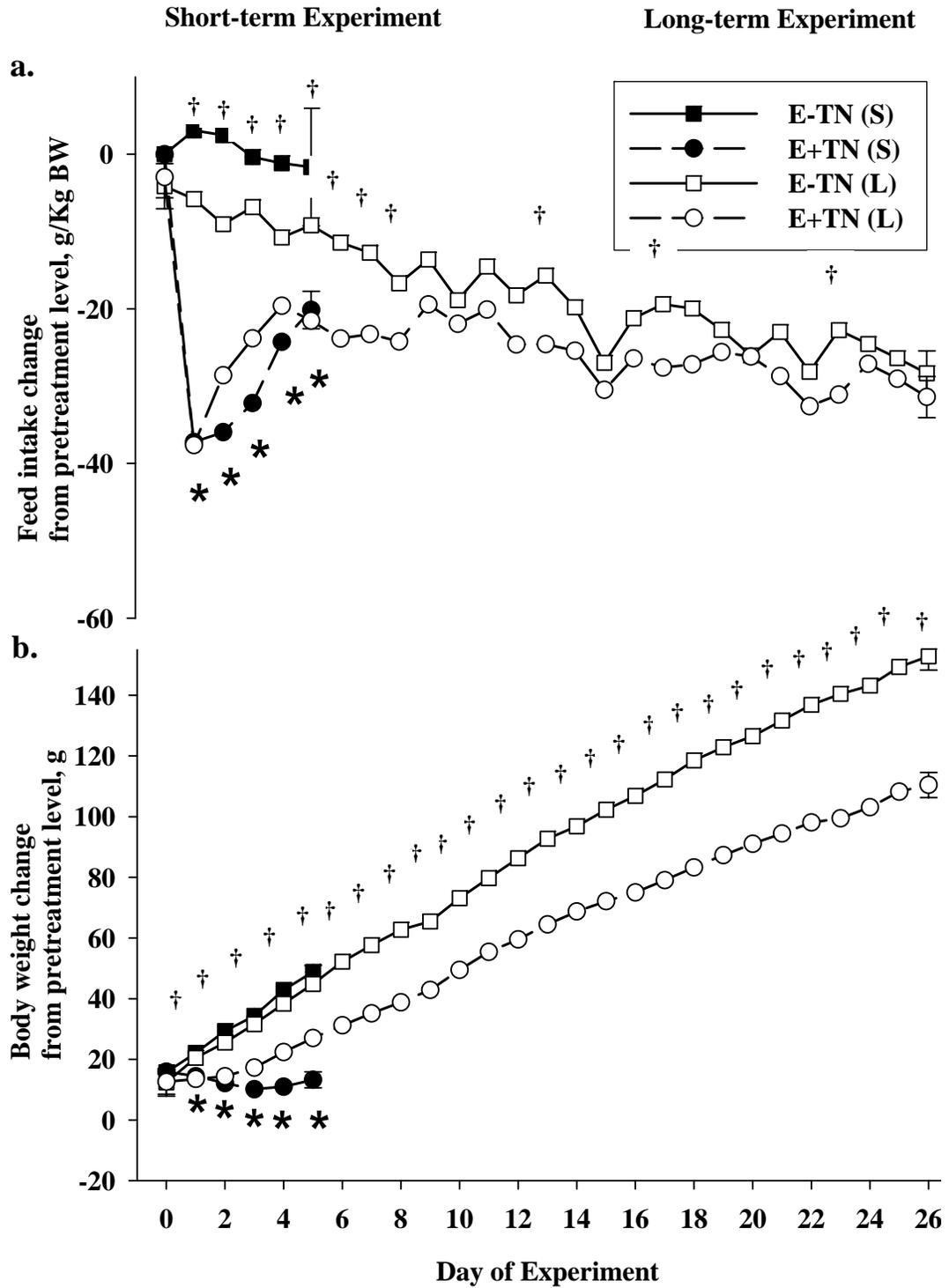


Figure 3. 2. Group average values for average daily feed intake (a), and daily body weight (b) of rats fed either E- or E+ diets during short- (S; 5 days) or long-term (L; 26 days) exposures at thermoneutrality. An asterisk (*) shows a significant difference ($p < 0.05$) between E- and E+ groups during the short-term experiment, whereas a dagger (†) shows a significant difference ($p < 0.05$) between E- and E+ groups during the long-term exposure.

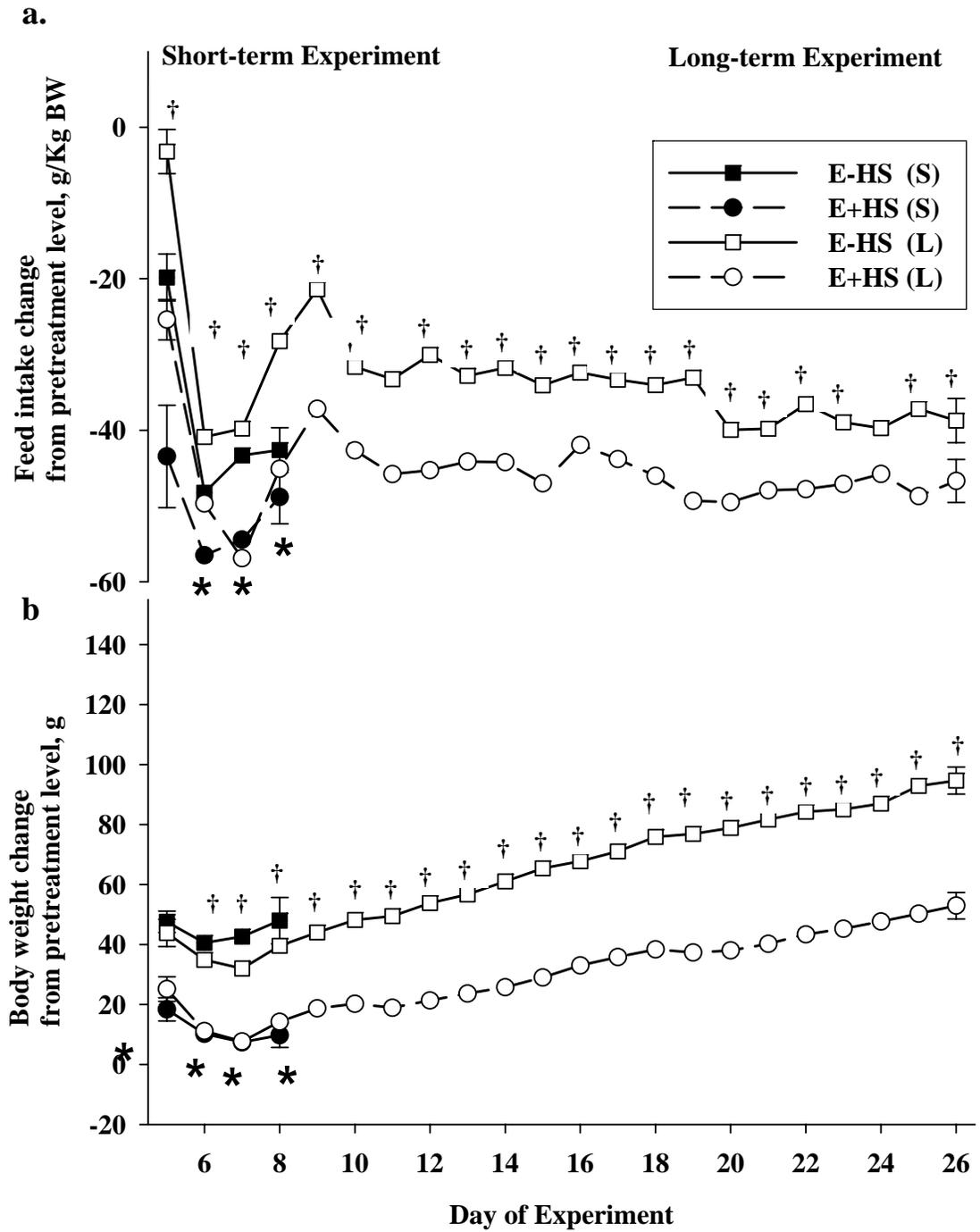


Figure 3. 3. Group average values for average daily feed intake (a), and daily body weight (b) of rats fed either E- or E+ diets during short- (S; 5 days) or long-term (L; 26 days) exposures at thermoneutrality. An asterisk (*) shows a significant difference ($p < 0.05$) between E- and E+ groups during the short-term exposure, whereas a dagger (†) shows a significant difference ($p < 0.05$) between E- and E+ groups during the long-term exposure.

Table 3. 1. Average values representing the growth slope for rats fed endophyte-free (E-) or endophyte-infected tall fescue (E+) diets for short-term period^a or long-term period^b under thermoneutral or heat stress conditions

Variable	Short-term period				Long-term period			
	E-	E+	SE ^c	p-Value	E-	E+	SE ^c	p-Value
Thermoneutral	6.70	-0.35	0.42	< 0.01	5.13	3.99	0.25	< 0.01
Heat stress	3.72	-0.23	0.85	< 0.01	3.09	2.12	0.26	> 0.05

^aThermoneutral – Experiment 1 – Days 1 to 5

Heat stress – Experiment 2 – Days 6 to 8

^bThermoneutral – Experiment 3 – Days 1 to 26

Heat stress – Experiment 3 – Days 6 to 26

^cStandard error of the mean

Table 3. 2. Group mean serum biochemistry values for rats fed endophyte-free (E-) or endophyte-infected tall fescue (E+) diets for 5 (short-term TN) or 26 days (long-term TN) under thermoneutral conditions

Variable	Short-term TN period				Long-term TN period			
	E-	E+	SE ^a	p-Value	E-	E+	SE ^a	p-Value
Glucose (mg/dL)	205.9	181.3	6.3	0.01	187.4	170.2	10.7	0.27
Urea nitrogen (mg/dL)	17.8	12.8	4.2	< 0.01	20.4	19.6	0.9	0.54
Cholesterol (mg/dL)	78.3	68.0	5.4	0.04	82.2	74.2	3.2	0.09
Alkaline phosphatase (U/L)	332.9	209.7	4.1	< 0.01	283.0	243.0	21.9	0.21
Prolactin (ng/mL)	13.0	6.1	0.9	0.02	22.6	12.0	1.7	< 0.01

^a Standard error of the mean

Table 3. 3. Average values of wet organ weights (g/Kg BW) from rats fed endophyte-free (E-) or endophyte-infected tall fescue (E+) diets for 5 days (short-term period) or 26 days (long-term period) under thermoneutral or heat stress conditions

Variable	Short-term period				Long-term period			
	E-	E+	SE ¹	p-Value	E-	E+	SE ^a	p-Value
<i>Thermoneutral conditions</i>								
Liver	44.6	38.4	0.9	< 0.01	39.2	36.9	0.9	0.07
Testis	8.5	10.1	0.5	0.03	7.7	8.8	0.4	0.04
<i>Heat stress conditions</i>								
Liver	35.8	31.2	0.5	< 0.01	34.5	30.7	0.9	< 0.01
Testis	9.4	10.5	0.5	0.10	8.3	10.2	0.5	< 0.01

^a Standard error of the mean

Table 3. 4. Group average serum biochemistry values for rats fed endophyte-free (E-) or endophyte-infected tall fescue (E+) diets for 3 (short-term HS) or 21 days (long-term HS) under heat stress conditions

Variable	Short-term HS period				Long-term HS period			
	E-	E+	SE ¹	p-Value	E-	E+	SE ^a	p-Value
Glucose (mg/dL)	135.2	133.2	9.3	0.88	184.0	152.6	10.7	0.05
Urea nitrogen (mg/dL)	16.0	17.2	1.0	0.42	18.0	16.8	0.9	0.36
Cholesterol (mg/dL)	77.8	68.0	5.0	0.18	81.8	73.0	3.2	0.06
Alkaline phosphatase (U/L)	312.8	262.6	20.8	0.10	253.6	187.2	21.9	0.04
Prolactin (ng/mL)	33.4	19.0	1.6	< 0.01	34.0	19.0	1.7	<0.01

^a Standard error of the mean

CHAPTER IV

GENOMIC ANALYSIS OF THE IMPACT OF FESCUE TOXICOSIS ON HEPATIC FUNCTION

ABSTRACT

Fescue toxicosis is caused by consumption of toxins produced by an endophytic fungus, *Neotyphodium coenophialum*, in tall fescue [*Lolium arundinaceum* (Schreb) Darbysh]. Microarray analysis was used to identify shifts in genetic expression associated with affected physiological processes to identify potential targets for future pharmacological/toxicological studies. Male rats (n = 24) were implanted with temperature transmitters which measure core temperature (Tc) every 5 min. Following an 8-d recovery, they were fed an endophyte-free (E-) diet for 5-d. During the following 5-d treatment period, rats were fed either an E- or endophyte infected (E+; 91.5 $\mu\text{g EV}\cdot\text{kg BW}^{-1}\cdot\text{d}^{-1}$) diets. At treatment end, rats were euthanized and liver was sampled. Feed conversion efficiency (FCE) was calculated for both treatment groups. Serum prolactin levels were measured using ELISA. Liver tissue RNA was reverse transcribed and hybridized to an oligonucleotide microarray chip. Microarray data were analyzed using a two-step ANOVA model, and validated by real-time PCR. Significant reductions in mean Tc, feed intake, FCE, body weight (BW), mass-specific liver weight, and serum prolactin

concentrations were observed in E+ rats. There was down-regulation ($p < 0.05$) of various genes associated with energy metabolism, growth and development, and antioxidant protection, as well as an up-regulation of genes associated with gluconeogenesis, detoxification, and biotransformation. This study demonstrated that even short-term exposure of rats to tall fescue endophytic toxins under thermoneutral conditions can result in physiological responses associated with altered gene expression within the liver.

2. INTRODUCTION

Intake of endophyte-infected (*Neotyphodium coenophialum*) tall fescue produces problems in animals that are collectively referred to as fescue toxicosis. The signs of fescue toxicosis include decreased feed intake, BW gain, milk production, and increased peripheral vasoconstriction, rough hair coat and hyperthermia during heat stress (Strickland et al., 1993). There are significant data gaps which underlie cellular and metabolic aberrations associated with this problem to limit development of pharmacological strategies. A novel approach to elucidate the altered cellular mechanisms associated with fescue toxicosis is to use microarray analysis (Jones et al., 2004).

The liver is the most important organ for xenobiotic metabolism and detoxification, and has been identified as a target organ of fescue toxicosis (Oliver, 1997). Oliver (1997) observed an increase in both glycogenolysis and urine glucose levels in cattle with fescue toxicosis. Piper et al. (1991) noted an increase in liver-specific

enzymes in long term E+ grazing animals, and a component of the fescue toxicosis response in rats is a decrease in liver weight (Eichen et al., 2001). Chestnut et al. (1992) noted that rats fed an E+ diet exhibited a decrease in liver weight which was observed in rats fed a control diet at the same caloric level (i.e., pair-fed). While it is likely that fescue toxicosis has both direct and indirect (i.e., reduced caloric intake) effects on hepatic function, the objective of this preliminary study was to determine the overall impact of fescue toxicosis on hepatic gene expression. It was hypothesized that short-term intake of an E+ diet at thermoneutrality would change hepatic gene expression associated with fescue toxicosis. This research represents one of the first attempts to determine changes in genetic expression associated with fescue toxicosis.

3. MATERIALS AND METHODS

Experimental design and evaluation of physiological responses

animals and treatment diet

Sprague-Dawley male rats (n = 12 per treatment; 42-d old) (Charles River Laboratories, Willington, MA), weighing 185 to 215 g, were maintained at 21°C (Ta) and 50 ± 5% humidity as previously reported (Spiers et al., 2005a). Feed and water were provided ad libitum throughout the study. Composition of control and treatment diets were as previously described, with the treatment diet adjusted to provide a specific amount of ergovaline (EV; the major ergot alkaloid associated with fescue toxicosis; Spiers et al., 2005a). Control (E-) seed contained 22 ppb of EV whereas E+ contained a concentration of 4,100 ppb on a dry matter basis as measured by HPLC (Detection limit =

50 ppb and CV = 7%; Rottinghaus et al., 1993). All procedures on animals followed the guidelines for humane treatment set by the University of Missouri Animal Care and Use Committee.

Treatment period and sample collection

The experimental schedule consisted initially of an 8-d recovery period after surgery for intraperitoneal implantation of telemetric temperature transmitters (Model VM-FH; Mini-Mitter Company, Inc., Bend, OR), followed by a 5-d pretreatment period (d -1 to -5), during which Tc, feed intake, and BW measurements were collected to establish a baseline for statistical analysis (Spiers et al., 2005a). During the 5-d duration of treatment (d 1 to 5), rats were fed either E- or E+ ($91.5 \mu\text{g EV}\cdot\text{kg BW}^{-1}\cdot\text{d}^{-1}$) diets while Tc, feed intake, and BW measurements were collected. At the end of study (d 6), rats were anesthetized and exsanguinated, and the liver was collected and weighed. A transverse section from the middle lobe of liver (~ 5 g) was fixed in 10% neutral buffered formalin for histological examination, and another portion (~ 5 g) was snap-frozen in liquid nitrogen for microarray experiments. Serum from the collected blood sample was frozen at -20°C for later use. Serum PRL concentrations were measured using ELISA, following manufacturer's protocol (Spi bio, Massy Cedex, France).

Histological examination of liver

Fixed liver sections were stained with hematoxylin and eosin (H & E) to study the histological changes and with periodic Acid-Schiff (PAS) to compare the levels of stored glycogen in E+ and E- rats. Images were captured at 400X magnification (Insight Color Camera, Sterling Heights, MI). A scoring system (one to four) was devised, with one denoting little or no vacuolation and four indicating marked vacuolation. A

vacuolation score was assigned to the liver sections by a board certified veterinary pathologist, without prior knowledge of their treatment group assignment.

Statistical analysis of physiological responses

Mean values for Tc, feed intake and BW were computed as a function of time and treatment group. The above parameters were analyzed by repeated measures analysis of variance (mixed procedure) of SAS (Littell et al., 1998) using treatment, time, and animal as class variables to determine overall treatment and time-related differences, and treatment X time interactions.

Microarray and real-time PCR analyses of genomic responses

Selection of experimental animals

In the present study, eight out-bred rats (four per treatment group) were used to study the genomic effects of fescue toxicosis. Because of the costs associated with microarray analyses, the four E+ rats showing the most pronounced evidence of fescue toxicosis were selected for microarray analysis. Selection was based on reduction in feed intake, FCE, liver weight, and Tc. Real-time PCR (RT-PCR) was used for all 24 rats to validate the microarray experimental results at the population level.

Microarray slide printing

Microarray slides were prepared by printing 1,353 Oligonucleotides (50 bases long) specific to rat liver (MWG Biotech AG, Ebersberg, Germany) on pan epoxy glass slides (MWG Biotech AG, Ebersberg, Germany) using a 16 pin robotic microarray printer. Each block in the microarray slide also contained: 10 *Arabidopsis thaliana* plant genes (Spot report oligoTM, Stratagene, Cedar Creek, TX) as external controls, 3X SSC as

negative controls, and rat Cot-1 DNA (Stratagene, Cedar Creek, TX) and poly A (Stratagene, Cedar Creek, TX) to detect non-specific hybridization.

RNA extraction

Total RNA was extracted from liver samples using RNeasy Midi Kit (Qiagen Inc., Valencia, CA), purified using DNase-1 (Ambion Inc., Austin, TX) and phenol, chloroform, isoamyl alcohol (25:24:1) and concentrated using microcon filters (Amicon Bioseparations, Bedford, MA) following the manufacturer's instructions. The RNA concentrations were measured using a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

cDNA preparation

Concentrated E+ or E- total RNA (15 µg) was reverse transcribed to cDNA using oligo dT (IDT DNA, Coralville, IA), random hexamers (IDT DNA, Coralville, IA), 50x amino-allyl dUTP (Sigma Chemicals, St. Louis, MO) and reverse transcriptase (StrataScript™ RT, Stratagene, La Jolla, CA). The resulting cDNA was purified using Microcon-30 filters (Millipore Corp., Bedford, MA) and conjugated either with Cy3 or Cy5 dyes separately by incubating in the dark for one hour. Conjugated oligonucleotides were separated from free, unconjugated dye using Qia-quick PCR purification kit (Qiagen, City, Valencia, CA), following manufacturer's instructions. The two cDNA samples, labeled with Cy3 and Cy5 dyes, were mixed together and dried.

Microarray hybridization

Dried cDNA pellet was resuspended with the hybridization mixture, which consisted of 1X 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.0), poly A (10 µg/µl) (Stratagene, Cedar Creek, TX), rat Cot-I DNA (3.2 µg/µl) 20X

standard saline citrate (SSC), (Stratagene, Cedar Creek, TX) and 10% sodium dodecyl sulfate (SDS). The slides were incubated in a water bath (65°C) for 12 to 16 h. Following incubation, the slides were rinsed in Wash Solution I (20X SSC, and 10% SDS) and then in Wash Solution II (20X SSC).

Microarray slide scanning and analysis

Microarray slides were scanned using GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA). Signal intensity of each spot from the scanned image was analyzed using Genepix Pro 3.0 software (Axon Instruments, Union City, CA). The spots were filtered based on their spot intensities, and flagged bad if the number of pixels that exceeded a background-related threshold (background intensity ± 1 SD) lowers than 55%. The spots were flagged, “not found” when the spot had less than six pixels above the background-related threshold, or if the spots overlapped the adjacent spots. The microarray data was stored in the BioArray Software Environment database (Saal et al., 2002).

Self-self hybridization

Six self-self hybridizations were performed to measure the variation in gene expression due to technical errors. In these, the same rat was used for different RNA isolations, labeling, and hybridizations. The slides were hybridized, scanned, and analyzed as above. Means and standard deviations for Cy5 and Cy3 ratios were calculated for the six hybridizations and a significant threshold was set as mean ± 2 SD, which was ± 1.34 . Genes with expression above or below this threshold were considered as up- or down-regulated, and used for further analysis.

Microarray analysis using ANOVA model

A loop design was followed for the microarray experiments. Intensity readings were transformed to log base two scales. The microarray data was analyzed using a two-step mixed linear model following the procedures of Kerr and Churchill (2002) and Wolfinger et al. (2001). The first step was normalization across genes, which identified and removed the variation caused by the array, pin, and dye effects and all of their first order interactions. The residuals from this model were used as the input for the second stage to model and remove the animal and dye effects and their interactions. The residual values from these analyses were the normalized data set. The use of ANOVA model allowed experimental effects to be estimated through the partitioning of the variations in the experiment and helped in inter-treatment comparisons without an internal reference.

First model (Normalization):

Where,

$$Y_{\text{adgp}} = \mu + A_{\text{a}} + P_{\text{p}} + D_{\text{d}} + (AP)_{\text{ap}} + (AD)_{\text{ad}} + (PD)_{\text{pd}} + \epsilon_{\text{adgp}}$$

Where,

Y_{adgp} : Log base two observed intensity of each experimental gene on the arrays

μ : Mean value across all genes and arrays

A_{a} : Array main effect

P_{p} : Pin, or panel, main effect

D_{d} : Dye main effect

$(AP)_{\text{ap}}$: Pin by Array interaction effect

$(AD)_{ad}$: Dye by Array interaction effect

$(PD)_{pd}$: Pin by Dye interaction effect

ϵ_{adgp} : Stochastic error term

The residual values from the normalization model were obtained by subtracting the fitted value for the effects from the Y_{agp} value. Variation due to pin and array effects significantly contributed to gene expression (25%).

Second model (Gene specific model):

$$R_{dgn} = \mu_g + (GA_n)_{gn} + (GD)_{gd} + \epsilon_{dgn}$$

Where,

μ_g : Mean value for gene g,

$(GA_n)_{gn}$: Random effect for gene g by animal A_n ,

$(GD)_{gd}$: Random effect for gene g by dye D_d and

ϵ_{dgn} : Stochastic errors obtained from the gene-specific models.

The second model of the microarray analysis consisted of a gene specific model for the output from the normalization model. Microarray analyses were computed using Proc Mixed Procedure in SAS (SAS Institute Inc, Cary, NC). A t-test was used to find differentially expressed genes, between treatment groups. The residual values obtained after the normalization model were also analyzed using a nonparametric Kruskal-Wallis method. Similar p-values were obtained for the differentially expressed genes using either method.

The GA values obtained from the ANOVA model analysis were further analyzed using Significance Analysis of Microarray (SAM) (Tusher et al., 2001) program. The

microarray data was permuted, using the following parameters: 2-class, unpaired, not log transformed, 1,000 permutations. A hierarchical tree was built with the results from SAM analysis using the TMev program (Eisen et al., 1998). The hierarchical tree was confirmed using bootstrap (Kerr and Churchill, 2002). The DAVID (Dennis et al., 2003) and Locuslink (<http://www.ncbi.nlm.nih.gov/LocusLink>) were used to find the gene annotation, and the functions of differentially expressed genes.

Microarray raw data files were deposited with the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>). The serial entry number for the microarray data is GSE2452 with the platform number being GPL 1786. Each hybridization, in the microarray experiment was given the following serial numbers: GSM46406, GSM46407, GSM46408, GSM46409, GSM46410, GSM46411, GSM46412, GSM46413, GSM46414, GSM46415, GSM46416, GSM46417, GSM46418, GSM46419, GSM46420, GSM46421, GSM46422, GSM46423, GSM46424, GSM46425, GSM46426, GSM46427, GSM46428, and GSM46429.

Quantitative real-time PCR

Real-time PCR was performed in a subset of eight representative genes for which the fescue toxicosis-induced changes in expression were considered statistically significant by microarray analysis and the pathways represented were of interest from the perspective of fescue toxicosis-induced hepatic effects. Table 4. 1 shows the gene-specific primers which were designed with the Primer Express program (Applied Biosystems, Warrington, UK) using default settings (primer T_m = 58 to 60°C, GC content = 30 to 80%, length = 9 to 40 nucleotides). Glyceraldehyde -3- phosphate

dehydrogenase (GAPDH) was used as the house keeping gene. The annealing temperature for the primers was standardized using gradient PCR and 3% agarose gels. The reaction mixture consisted of 2X SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK), 50 nM forward and reverse primers and 0.1 ng/ μ l cDNA. Thermal cycling was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems Warrington, UK) under factory default conditions (50°C, 2 min; 95°C 10 min; and 40 cycles at 95°C, 15 sec; 60°C, 1 min). Each gene was measured in triplicates and the formation of single PCR product was confirmed using dissociation curves. The normalized C_t values for each gene in all the 12 rats of each treatment group were averaged and the relative percent change in the expression of the gene in E+ rats was calculated. Student's t-test was used to statistically compare the C_t values between E+ and E- rats.

4. RESULTS

Physiological responses to fescue toxicosis

Core body temperature

A previously reported (Spiers et al., 2005a), circadian rhythm for the rat core body temperature was also observed in this study. Consumption of E+ diet resulted in a time related decrease ($p = 0.04$) in T_c (Figure 4. 1) in the E+ fed treatment group, with the temperatures decreasing ($p < 0.05$) from d 4. In contrast, no significant decrease in T_c was observed in E- rats during the treatment period.

Feed intake and conversion

Average daily feed intake was not affected in E- rats throughout the treatment period. In contrast, there was a rapid decrease (31.73 g/kg BW; 55%) in average daily feed intake within the first 24 h on the E+ diet ($p < 0.01$). Although a partial recovery was observed at the end of the 5-d treatment period, the average daily feed intake was lower ($p < 0.001$) in E+ compared to E- rats (55.97 ± 1.93 g/kg BW versus 65.86 ± 1.93 g/kg BW).

No significant changes were observed in the mean FCE in E- rats during the treatment period. However, average FCE dropped in the E+ rats ($p < 0.01$) immediately after the first day of treatment (-14.97 g feed/kg BW). By the end of d 5, there was an increase ($p = 0.02$) in average FCE in E+ rats compared to d 1 during the treatment period. Despite this improvement, average FCE was still much lower in the E+ treatment group (-11.09 g feed/kg BW) compared to the E- treatment group (32.24 g feed/kg BW) at the end of treatment ($p < 0.01$).

Body and organ weights

Mean BW of E+ rats was decreased ($p < 0.05$) on d 4 and 5 compared to E- rats, with average BWs of E- and E+ rats being 333.26 ± 8.11 and 295.57 ± 8.11 g, respectively, at the conclusion of the study. Mean liver weights were compared between E+ and E- rats as a preliminary measure of the effect of fescue toxicosis. Average wet weights (g) per kg BW of liver decreased in E+ (38.43 ± 0.15) compared to E- (44.55 ± 0.15) rats ($p < 0.01$).

Additional effects

Liver sections stained with hematoxylin and eosin showed differences with respect to the amount of cytoplasmic vacuolation in E+ rats compared to E- rats (Figure 4. 4). Number of vacuoles differed ($p < 0.01$) in E- rats (2.58) compared to E+ (1.67) rats in the hematoxylin and eosin stained hepatic sections. Qualitative staining for glycogen with PAS suggested decreased stores of glycogen in the livers of E+ relative to E+ rats (Figure 4. 4). As expected, the mean serum PRL concentration was lower ($p = 0.02$) in E+ rats (6.05 ± 0.51 ng/ml) as compared to the E- rats (12.95 ± 1.28 ng/ml), verifying the existence of fescue toxicosis in the E+ treatment group.

Genomic responses to fescue toxicosis

Hepatic microarray results

Average mean ± 2 SD intensity values (± 1.34) of the six self-self hybridizations were used as the threshold to exclude the genes that exhibited small changes in gene expression, but were identified as significant ($p < 0.05$) because of unusually small variance. Microarray analysis, using the SAM program showed 122 genes being differentially expressed with a false discovery rate (FDR) of less than 32%. The functions of 19 differentially expressed genes are unknown (data not shown). These genes are shown in Tables 4. 2a to 4. 2f. A large proportion of these differentially expressed genes (93) were down-regulated in the E+ treatment group, as compared to E- treatment group, with the expression of the remaining 29 genes being up-regulated in the E+ rats relative to the E- group. Hierarchical clustering showed the differentially

expressed genes in the rat hepatic tissue (Figure 4. 5) and bootstrap testing supported the hierarchical tree results with 100% confidence. Important pathways represented by the differentially expressed genes included energy metabolism, growth and development, detoxification and antioxidant mechanisms, as well as stress response, immune function, cell integrity and transportation and other cellular processes.

Quantitative real-time PCR

Quantitative real-time PCR was used to confirm the validity of the microarray analysis. The RT-PCR results confirmed the microarray data for seven of the eight selected genes in the 24 rats. In the microarray experiments, tryptophan 2,3-dioxygenase gene (FDR = 21.53) was upregulated by 162% when compared to E- rats, whereas in RT-PCR, it was upregulated by 360% ($p < 0.01$). The gene expression of cytochrome P450 2c13 (FDR = 27.68) was upregulated by 169% in E+ rats in the microarray results, whereas in RT-PCR it was upregulated by 338% ($p < 0.01$). Similarly the expression of phosphoenol pyruvate carboxykinase (FDR = 9.68; 202%), fructose-1,6- biphosphatase 1 (FDR = 27.68; 185%) and arginase (FDR = 9.68; 208%) genes were upregulated in E+ in microarray experiments. Similar expression was observed in RT-PCR experiment with a% change of 224 ($p = 0.03$), 364 ($p = 0.01$) and 470 ($p < 0.01$), respectively. Glutathione synthetase (FDR = 31.64) and heme oxygenase-1 (FDR = 31.65) genes were down-regulated by 172 and 173% in E+ rats in microarray analysis and in RT-PCR they were down-regulated by 142 ($p = 0.05$) and 131% ($p = 0.06$), respectively. Catalase, (FDR = 27.68) which was upregulated in E+ rats in microarray results (169%) was down-regulated in RT-PCR (186%; $p = 0.04$).

5. DISCUSSION

Physiological responses associated with fescue toxicosis

Fescue toxicosis in cattle is associated with a number of physiological responses, including altered thermoregulation (Spiers et al., 1995; Stuedemann et al., 1985), decreased feed intake and average daily gain (Nihsen et al., 2004), inefficient feed conversion (Bush et al., 2001) and hypoprolactinemia (Schillo et al., 1988; Cross et al., 1995). All except the last two responses have been previously reported in the rat model for fescue toxicosis (Spiers et al., 2005a). However, all of these responses were observed in the present study after only 5-d of exposure to an E+ diet at thermoneutrality (Figures 4. 1 to 4. 3).

Toxins associated with fescue toxicosis have high affinities for α -adrenergic, dopaminergic and nearly all subtypes of serotonergic receptors (Oliver, 2005). Interactions of E+ toxins with these receptors increase vasoconstriction (Oliver, 2005), decrease feed intake, gastrointestinal secretions, segmental activity, nutrient absorption (Oliver, 1997), and suppress prolactin secretion from the anterior pituitary (Elsasser and Bolt, 1987).

Genomic responses associated with fescue toxicosis

It was hypothesized that short-term intake of an E+ diet at thermoneutrality would result in changes in hepatic gene expression that is associated with the pathogenesis of fescue toxicosis. The microarray and real-time PCR results demonstrated that the expression of genes belonging to several important physiological pathways, including energy metabolism, growth and development, detoxification and antioxidant activity, as

well as stress response and immune function, were altered by E+ treatment. Genomic responses to the direct hepatic effects of endophytic toxins or, secondarily, to indirect effects of fescue toxicosis on the liver (e.g., decreased caloric intake) are of interest to scientists looking for novel prophylactic and therapeutic approaches to endophyte-infected fescue problems in livestock. A careful analysis of these genomic effects and their relationship to the observed physiological responses should provide additional insight into the pathophysiology of fescue toxicosis.

Energy metabolism

As previously noted, prominent physiological responses to fescue toxicosis include reductions in feed intake, average daily gain, and FCE. Consistent with earlier results (Spiers et al., 2005a), all of these were also observed in the present study (Figures 4. 2 and 4. 3). The hepatic effects of these physiological responses were demonstrated by both the decreased mean relative liver weight of E+ compared to E- rats, which was also observed by Chestnut et al. (1992) in rats pair-fed an E- diet, and by the histological changes in the E+ livers, which most likely reflected the depletion of hepatocellular stores of glycogen and lipids (Figure 4. 4). Consistent with decreased caloric intake and FCE, genes involved in lipid and carbohydrate metabolism (e.g., liver-specific Bhlh-Zip transcription factor-7, ATP citrate lyase, and glucuronidase beta) were down-regulated by E+ treatment (Table 4. 2a). Fescue toxicosis-induced up-regulation of genes involved in gluconeogenic pathways, such as phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase (FBP) (Table 4. 2a), indicated an adaptive response to lower caloric intake during exposure to E+ diets. In addition, several genes associated with protein catabolism in other pathways, including arginase, 4-hydroxy phenyl pyruvic acid

dioxygenase (HPPD) and epoxide hydrolase 1, were also up-regulated in E+ rats (Tables 4. 2b and 4. 2c), supporting a relationship between protein catabolism for gluconeogenesis in E+ rats. Other studies have shown that during starvation, genes involved in gluconeogenesis (e.g., PEPCK; Perez et al., 1998), and protein catabolism (e.g., HPPD; Endo, 1999), are up-regulated.

Several important genes involved in mitochondrial activity (i.e., ATP production) were also down-regulated in E+ compared to E- rats (Table 4. 2a). The gene for mitochondrial transcription factor A encodes for a key activator of mitochondrial transcription, as well as a participant in mitochondrial genome replication. Mitochondrial transcription factor A is required for accurate and efficient promoter recognition by mitochondrial RNA polymerase (Murthy et al., 1981). Mitochondrial ATP synthase is mainly involved in the production of ATP from ADP in the presence of a proton gradient across the mitochondrial membrane (Yoshida et al., 2001). Down-regulation of these important genes, as well as ATPase 3 (proteosome 26S subunit) and thioredoxin reductase 2 (Table 4. 2c), in E+ rat livers could impair ATP production and would help explain the reduced FCE and hypothermia at thermoneutrality observed in endophyte-exposed animals.

Growth and development

Despite the apparent recovery in average daily feed intake observed in E+ rats (Figure 4. 2), average daily gain was consistently lower throughout the treatment period in endophyte-exposed rats (Figure 4. 3). This lack of recovery in average daily gain in rats fed the E+ diet reflected the effects of endophytic toxins, decreased caloric intake, and reduced FCE on E+ rats. The somatomedins or insulin-like growth factors (IGF)

mediate many of the growth-promoting effects of growth hormones. In association with reductions in FCE and average daily gain, genes for IGF-1, insulin-like growth factor binding protein 2 (IGFBP-2), and other genes involved in body growth and development, such as *erv1*-like growth factor (hepatopoietin), growth response protein and eukaryotic translation initiation factor 4E binding protein-1, were down-regulated in E+ as compared to E- rats (Table 4. 2b).

Apoptosis or programmed cell death occurs during development and aging and can be beneficial. However, excessive apoptosis can result in tissue damage. In the present study, genes which protect the cell from excessive apoptosis, such as IGF-1 (Mason et al., 2000), IGFBP-2, inhibitor of apoptosis protein-1 (Verhagen et al., 2001), heme oxygenase 1 (HO-1) (Brouard, 2000), and CCAAT/enhancer binding protein, were down-regulated in E+ compared to E- rats (Table 4. 2b). Down-regulation of these genes has the potential to increase the rate of apoptosis in E+ exposed hepatic tissue.

Detoxification and antioxidant activity

Hepatic biotransformation of xenobiotics (Phase I metabolism) and the subsequent conjugation of the resulting metabolites into compounds that can be excreted from the body (Phase II metabolism) are the primary hepatic processes involved in detoxification. Phase I metabolism frequently involves the CYP system, which is comprised of many mixed function oxidative enzymes. Cytochrome P450 enzymes are membrane-bound proteins that catalyze oxidation of many endo- and xenobiotic compounds. CYP 2E1 and CYP 2C13 play important roles in xenobiotic metabolism (Oguri and Yamada, 1994), and livers from E+ rats showed up-regulation of the genes for these two enzymes when compared to the livers from E- rats (Table 4. 2c). The gene for

epoxide hydrolase-1, another important enzyme involved in detoxification (Reid et al., 2002) was also up-regulated in the livers of E+ rats (Table 4. 2c).

Increased expression of these detoxifying genes might assist in the metabolism of endophytic ergot alkaloids, thereby reducing some of the clinical signs of fescue toxicosis, and this proposed detoxification could be one explanation for the apparent recovery in feed intake observed in E+ rats in the present study (Figure 4. 2). Others studies have noted CYP induction following exposure to ergot alkaloids and have proposed a role for this enzyme system in the detoxification of ergot alkaloids. Zanzalari et al (1989) observed a 70% increase in hepatic mixed-function oxidase activity in E+ ewes after an 11-d endophyte-infected fescue feeding trial. Moubarak and Rosenkrans (2000) observed hydroxylation of ergotamine by a CYP isoform CYP3A in beef hepatic microsomes, and Ball et al. (1992) observed the role of CYP3A in the initial metabolism of ergot alkaloids via deethylation in human liver microsomes.

Interestingly, up-regulation of genes belonging to the CYP system can also be associated with decreased feed intake. Bauer et al. (2004) noted that various CYP isoforms were differentially expressed in murine livers during starvation. Therefore, it is likely that the CYP gene up-regulation observed in E+ rats in the present study could be the result of both endophytic toxin exposure, as well as a reduction in caloric intake.

Production of unstable intermediate metabolites during Phase I oxidation often leads to the production of free radicals (Scanlan, 2001). These reactive intermediates from Phase I metabolism undergo conjugation (e.g., sulfation, glucuronidation, glutathionation, methylation and acetylation) mediated by Phase II enzymes to produce extremely hydrophilic compounds which can be excreted (Scanlan, 2001). Glutathione

synthase is involved in a variety of biological functions, including protection of cells from oxidative damage by free radicals, detoxification of xenobiotics, and membrane transport (Grant et al., 1997). A variety of other enzymes are also involved in preventing oxidative damage in the liver. Heme oxygenase is a microsomal enzyme that catalyzes the oxidation of heme to the antioxidant molecules, biliverdin (Maines, 2000). Propyl 4-hydroxylase alpha neutralizes superoxides (Aravind et al., 2001), and thioredoxin reductase-2 reduces disulfide in oxidized thioredoxin to a redox-active disulfide. Decrease in the disulfide reducing activity of thioredoxin reductase-2 leads to a large increase in the NADPH oxidase activity, thereby producing more free radicals (Arner et al., 1995).

Microarray analysis in the present study showed that the genes involved in glutathione biosynthesis and glutathione synthetase were down-regulated in E+ rat liver (Table 4. 2c). Additional genes having antioxidant activity, such as HO-1, thioredoxin reductase-2, propyl 4-hydroxylase alpha subunit, and peroxiredoxin-3 were also down-regulated in E+ compared to E- rats (Table 4. 2c).

Down-regulation of the genes for antioxidants in the present study could lead to the accumulation of free radicals within the cell and is consistent with the results of earlier studies. Lakritz et al. (2002) observed lowered blood levels of glutathione and feed intake during fescue toxicosis in cattle under heat stress conditions. Oliver (1997) observed a decrease in the mean plasma antioxidant capacity in E+ steers seven times below that of E- animals. Oliver (1997) hypothesized that the reactive oxygen species might contribute to the inflammatory response in E+ fed animals.

It is unclear whether the down-regulation of genes associated with antioxidant activity in the liver in E+ rats is a direct effect of endophytic toxins or is secondary to a reduction in antioxidant precursors associated with decreased feed intake and FCE. Regardless, decreased antioxidant activity in the liver of endophyte-exposed animals is a possible mechanism for some of the clinical signs associated with fescue toxicosis. This potential for fescue toxicosis-associated oxidative stress is enhanced if Phase I enzymatic activity is high while the activities of Phase II enzymes and other hepatic antioxidants are low in endophyte-exposed animals (Scanlan, 2001). Therefore, the up-regulation of genes for Phase I enzymes and the down-regulation of genes involved in antioxidant activity in fescue toxicosis could lead to the accumulation of free radicals within the cells due to the impairment of cellular antioxidative mechanisms.

Stress response

Acute phase proteins are serum proteins that increase in animals under stress conditions (Lopez-Hellin et al., 2005). Genes involved in acute phase response such as bile acid coenzyme A, bile acid coenzyme ligase A, and coagulation factor-2 were up-regulated in the livers of E+ rats, as compared to the livers of E- rats (Table 4. 2d). Heat shock proteins, which are usually up-regulated in response to stress in order to protect cellular proteins (Gonzalez and Manso, 2004), were actually down-regulated in the present study in E+ compared to E- rats. These down-regulated heat shock protein genes in E+ rats included small stress protein, crystalline alpha B, and serine proteinase (Table 4. 2d). Fescue toxicosis has been reported to increase rat susceptibility to various stressors. In two separate studies, Filipov et al. (1999a, b) observed greater susceptibility of E+ mice to subcutaneous injections of cytokines and greater susceptibility of cattle

grazing on E+ pastures to the effects of lipopolysaccharides than their respective E- cohorts.

Immune function

Several genes associated with immune function, such as tubulin beta-5, scavenger receptor class B member 1, interferon beta 1, interleukin receptor accessory protein, and tubulin tyrosine ligase were down-regulated in the livers of E+ rats compared to the livers of E- rats (Table 4. 2e). Altered immune responses and inflammatory changes are commonly observed in fescue toxicosis (Filipov et al., 1999). Rodents fed E+ toxins exhibited a lowered red and white blood cell counts, reduced response to mitogens, and increased T suppressor cell numbers in spleen compared to E- rats (Dew et al., 1990).

Cellular integrity, transportation, and other processes

Twenty-five genes having to do with cell integrity and transportation, as well as another 25 genes involved in other cellular processes, were differentially expressed in the livers of rats fed E+ diets (Tables 4. 2f and 4. 2g). Of these 50 differentially expressed genes, only seven genes involved in structural and membrane transport processes were up-regulated. Given the relative lack of documentation of the effects of fescue toxicosis on these pathways, it is difficult to comment at length on the possible role of these genes in the pathogenesis of fescue toxicosis. However, future studies with pair-fed animals will help to determine whether the differential hepatic expression of these genes is related to the direct effects of endophytic toxins or to secondary effects on the liver associated with the reduction in caloric intake.

6. CONCLUSION

The present study provides information regarding specific hepatic genes which are differentially expressed in rats following intake of E+ diets at thermoneutrality. It has also helped to elucidate the cellular mechanisms involved in the pathogenesis of fescue toxicosis. Of particular interest are the pathophysiological roles of reduced caloric intake and FCE, as well as oxidative stress, in the development of signs of fescue toxicosis. The results of this study emphasize the importance of the inclusion of pair-fed treatment groups in future studies to differentiate the direct hepatic effects of endophytic toxins from the secondary effects on the liver that are associated with decreased feed intake. Future studies should be designed to identify differentially expressed genes during various time periods after exposure to endophyte-infected fescue and heat stress, in order to determine specific markers to be targeted for prophylactic and/or therapeutic interventions.

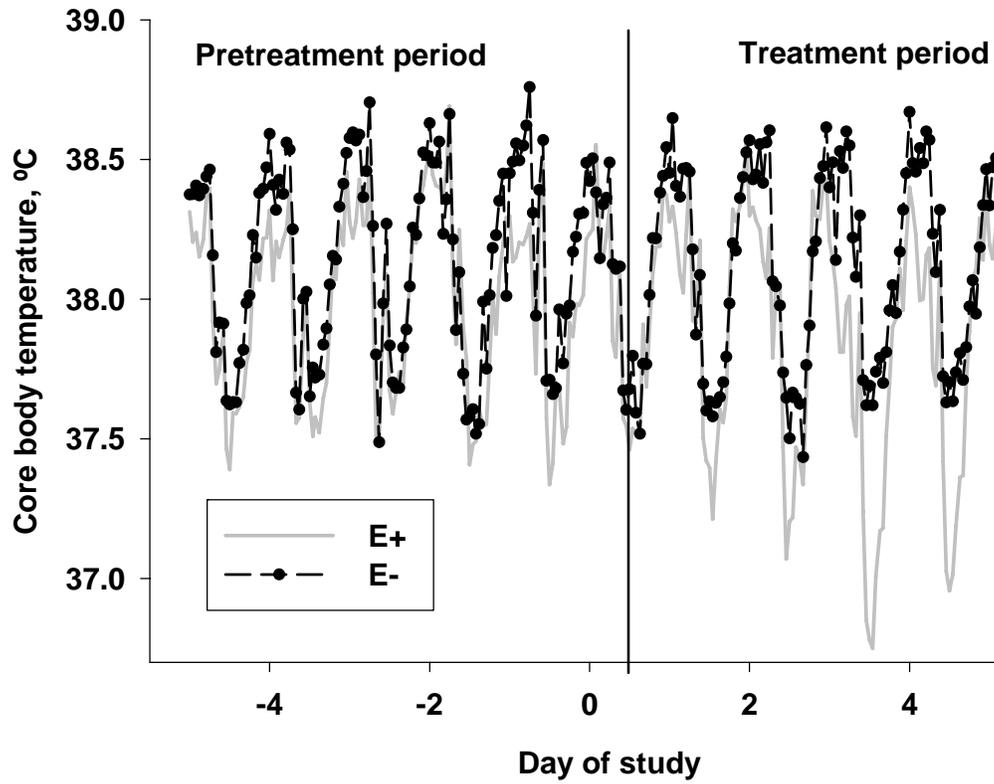


Figure 4. 1. Average core body temperature of endophyte-infected diet (E+) and endophyte-free diet (E-) rats during pretreatment and treatment periods as a function of time. The graph shows the circadian rhythm in core body temperature with peaks at midnight (rats being nocturnal).

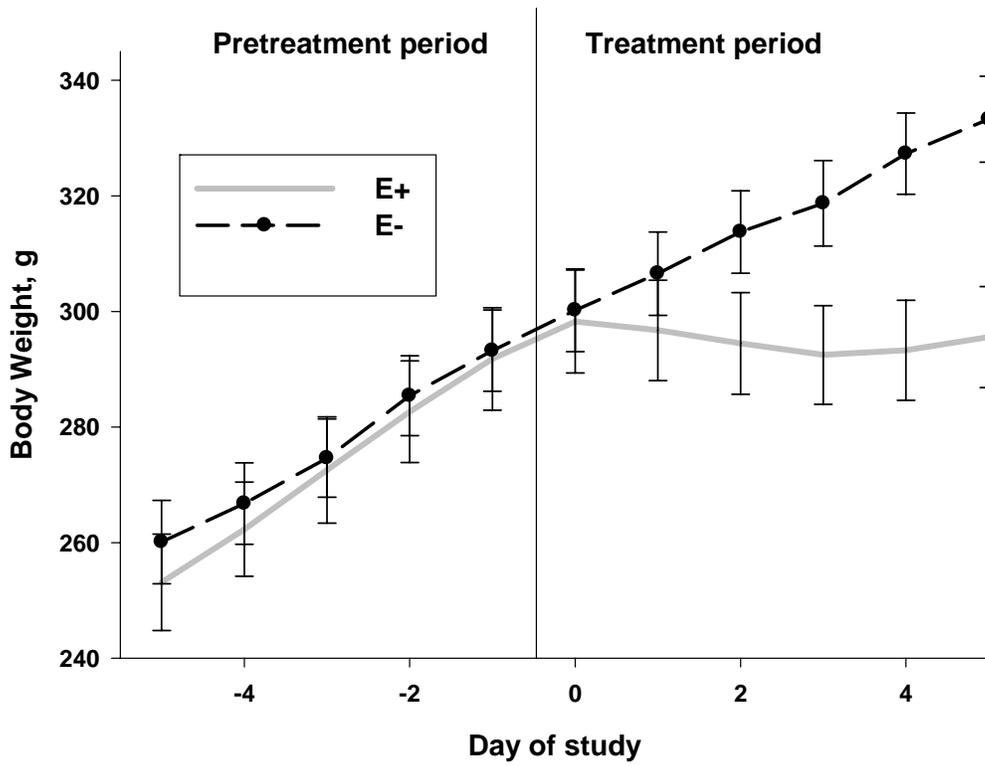


Figure 4. 2. Average daily BW of endophyte-infected diet (E+) and endophyte-free diet (E-) rats during pretreatment and treatment periods. Vertical lines on the first and last values indicate ± 1 SE.

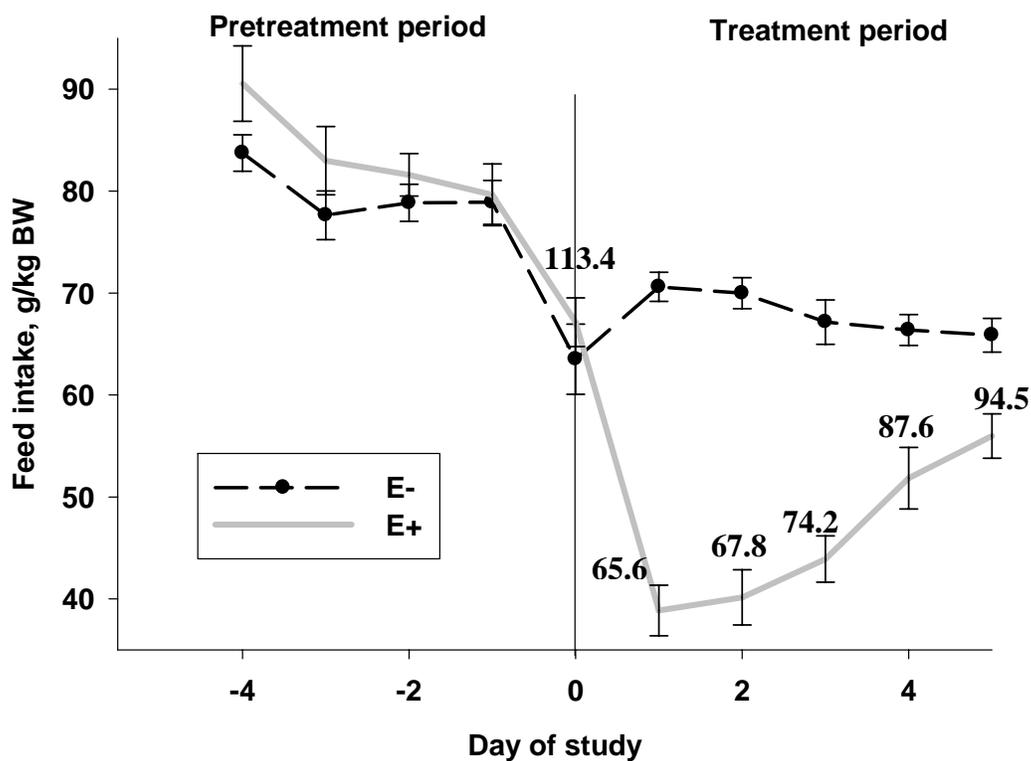


Figure 4. 3. Average daily feed intake of endophyte-infected diet (E+) and endophyte-free diet (E-) rats during pretreatment and treatment periods. The numbers on lines represent amount of ergovaline consumed (ppm) by E+ rats during the 5-d treatment period. Vertical lines on the first and last values indicate ± 1 SE.

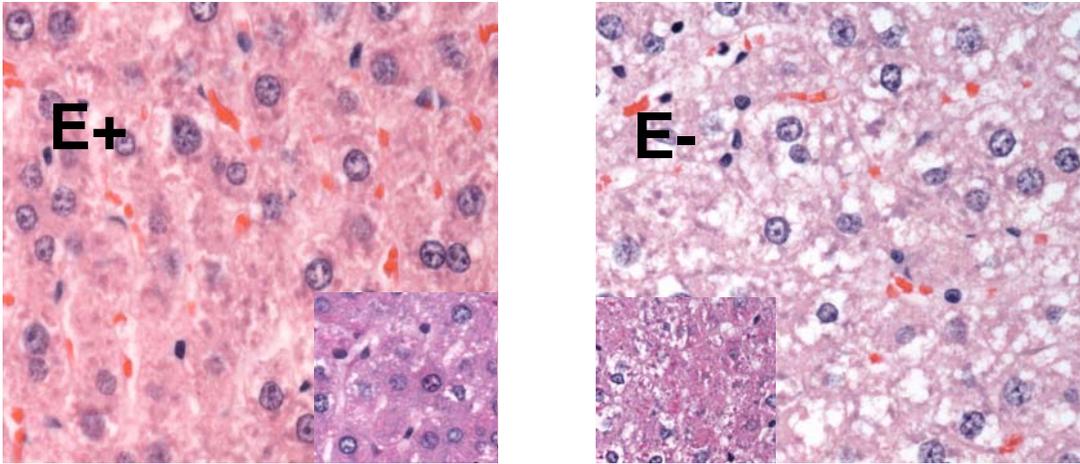


Figure 4. 4. Histological sections of liver stained with H&E showing vacuolation (fat globules) in the midzonal to periportal hepatocytes in endophyte-free diet (E-) compared to endophyte-infected diet (E+) rats. Nuclei are stained blue-black. Cytoplasm is stained with varying shades of pink. RBC is stained orange/red. Insets show the PAS stained liver sections indicating lower glycogen levels in E+ rats compared to E-. (Magnification 400X).

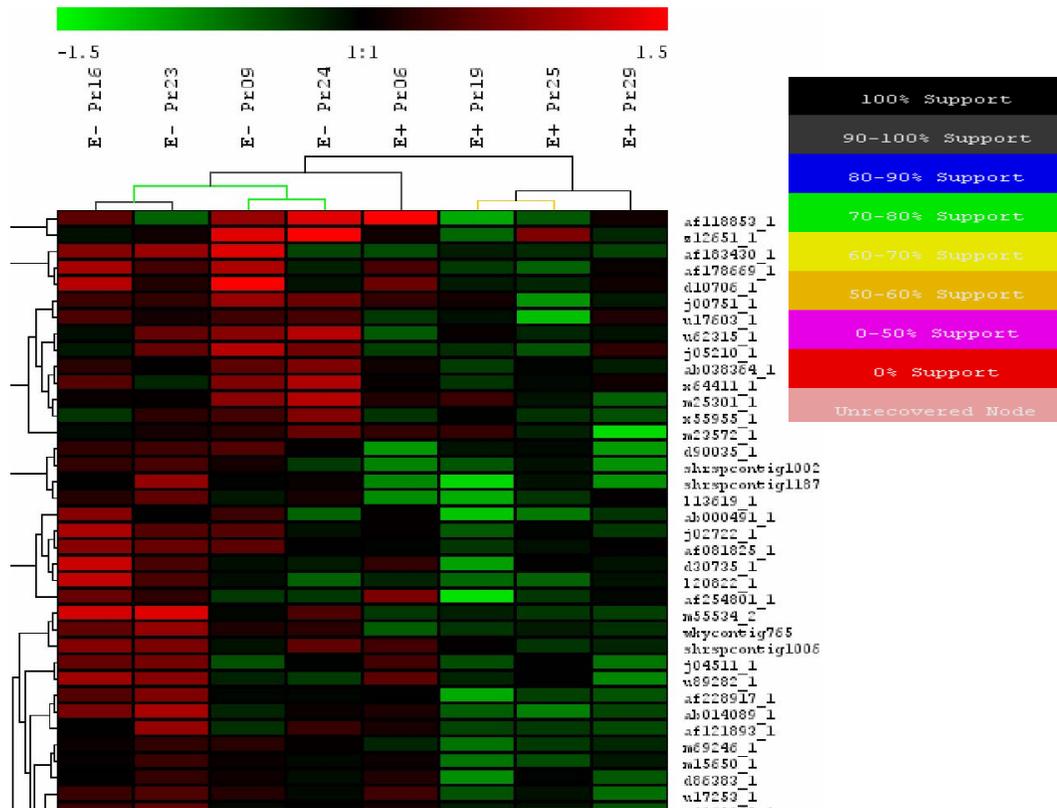


Figure 4. 5. Hierarchical tree showing the differentially expressed genes in endophyte-infected diet (E+) and endophyte-free diet (E-) rats at thermoneutrality, with the similarly expressed genes clustered together. The green blocks represent the down-regulated genes, red blocks represent up-regulated genes, and black blocks represent the genes which are not expressed. Gene accession numbers are shown on the right side of the tree with rat numbers and treatment names at the top.

Table 4. 1. Details of primers used for RT-PCR

Gene	Genbank No.	Forward primer	Reverse primer
CYP2C13	NC003280	AAAGAATGTTACTCAGTCTTCACAAACC	ACGACATGGTAATGGCCTTTTT
PEPCK	K03248	ACAGGCAAGGTCATCATGCA	TGCCGAAGTTGTAGCCAAAGA
Arginase	M17931	CCGCAGCATTAAAGGAAAGCT	TCCCCGTGGTCTCTCACATT
Tryptophan 2,3-dioxygenase	M55167	GAAAACAATGACTGCCTTGGA	GCAGCCGGAAGTGAAGACTCT
Fructose- 1,6- biphosphatase 1	J04112	CCTGCCAACAAAGAAAAATCCA	CGTAAGCGATGGGATTGCA
GAPDH	NM023964	TGCCAAGTATGATGACATCAAGAAG	AGCCCAGGATGCCCTTTAGT
Heme oxygenase 1	NP034572	CGCATGAACACTCTGGAGATGA	AGGCGGTCTTAGCCTCTTCTG
Glutathione synthetase	I38615	ATGGAGAAATAGGCAGTTCATGTG	CCGGCCCAGATCTCCTCTA
Catalase	NM012520	CCCAGAAGCCTAAGAATGCAA	GCTTTCCCTTGGCAGCTATG

Table 4. 2. List of significantly differentially expressed genes in E+ rat liver after the 5-d treatment period ^a

2a: Genes associated with energy metabolism

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
Phosphoenolpyruvate carboxykinase, cytosolic	PEPCK	k03248	Gluconeogenesis; Lipid metabolism; Phosphoenolpyruvate carboxykinase activity	202, Up	9.68	0.08
Fructose-1,6- biphosphate 1	Fbp1	j04112	Carbohydrate metabolism; Fructose-biphosphate activity; Gluconeogenesis	185, Up	9.68	0.04
Liver-specific bHLH-Zip transcription factor 7	Lisch7	af119667	Regulation of lipid metabolism	67, Down	31.65	0.06
ATP citrate lyase	Acly	j0 5210	Acetyl-Co A biosynthesis; Lipid biosynthesis	58, Down	31.64	0.04
Glucuronidase, beta	Gusb	M 13962	Carbohydrate metabolism	70, Down	31.65	0.01
Mevalonate pyrophosphate decarboxylase	Mpd	u53706	Cholesterol biosynthesis; Sterol biosynthesis	57, Down	31.65	0.05
Transcription factor A, mitochondrial	Tfam	ab014089	DNA binding; regulation of transcription; DNA-dependent	58, Down	31.65	0.01
ATP synthase	Atp5b	m 25301	ATP biosynthesis	68, Down	31.64	0.01
Proteasome (prosome, macropain) 26S subunit, ATPase 3	Psmc3	u77918	Mitochondrial inner membrane; nucleotide binding	57, Down	31.64	0.02
Proteasomal ATPase (SUG1)	LOC81827	ab000491	Ubiquitin dependent proteolysis	51, Down	31.64	0.02

2b: Genes associated with growth and development

Insulin-like growth factor binding protein 2	Igfbp2	J0 4486	Growth factor binding; regulation of cell growth	70, Down	31.65	0.06
Insulin-like growth factor 1	Igf1	m15650	Apoptosis inhibitor activity; growth factor activity; neurogenesis; organogenesis	68, Down	31.64	0.02

Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
Eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1	u05014	Insulin receptor signaling pathway; protein binding	63, Down	31.65	0.05
Growth response protein (CL-6)	LOC64194	ll 3619	Growth regulation	65, Down	31.65	0.03
Growth factor, erv1 -like	Gfer	d30735	Growth factor activity	53, Down	31.65	0.01
Interferon-related developmental regulator 1	Ifrd1	j0 4511	Neurogenesis	64, Down	31.64	0.01
CCCTC-binding factor (zinc finger protein)	Ctcf	af133731	Neurogenesis	66, Down	31.65	0.06
Ngfi-A binding protein 1	Nab1	u17253	Regulation of transcription; DNA-dependent	62, Down	31.64	0.08
Neural precursor cell Sequence-specific single-stranded-DNA-binding protein	Nedd8	af095740	Regulation of transcription from Pol II promoter	58, Down	31.64	0.06
	Ssdp	af121893	Transcription regulator activity	61, Down	31.65	0.06
RNA binding motif protein 10	Rbm10	d83948	Transcriptional activity	61, Down	31.64	0.08
CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	m 57235	Anti-apoptosis; transcription regulation	58, Down	31.65	0.01
Hepatocyte nuclear factor 3, alpha	Hnf3a	x55955	DNA-dependent; transcription factor activity	57, Down	31.65	0.06
Inhibitor of apoptosis protein 1	Birc2	af183430	Anti-apoptosis	48, Down	31.65	0.01
Arginase	Arg	m17931	Protein catabolism	208, Up	9.68	0.04

2c: Genes associated with detoxification and antioxidant activity

4-Hydroxyphenylpyruvic acid dioxygenase	Hpdd	af082834	Oxidoreductase activity; Protein catabolism	152, UP	21.53	0.03
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Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
NADH dehydrogenase (ubiquinone protein 6)	Ndufs6	l38437	Mitochondrion; Oxidoreductase activity	149, UP	27.68	0.04
Epoxide hydrolase 1	Ephx1	m15345	Oxidoreductase activity; Proteolytic activity	163, UP	27.68	0.03
Catalase	Cat	m11670	Electron transport; Oxidoreductase activity	169, UP	27.68	0.06
Cytochrome P450 2c13	Cyp2c13	j02861	Electron transport; Oxidoreductase activity	169, UP	27.68	0.02
Tryptophan 2,3-dioxygenase	Tdo2	m55167	Oxidoreductase activity; Tryptophan metabolism	162, UP	21.53	0.04
Cytochrome P450 2E1	Cyp2E1	j02627	Electron transport; Oxidoreductase activity;	221, UP	27.68	0.05
Thioredoxin reductase 2	Txnr2	af072865	Electron carrier activity; Oxidoreductase activity	58, Down	31.65	0.02
Peroxisome oxidoreductase 3	Prdx3	af106944	Oxidoreductase activity	73, Down	31.65	0.03
Nitric oxide synthase 3	Nos3	aj249546	Nitric oxide biosynthesis; Oxidoreductase activity	69, Down	31.65	0.03
Heme oxygenase 1	Hmox1	J0 2722	Oxidoreductase activity; Phospholipid metabolism	58, Down	31.65	0.03
Glutathione synthetase	Gss	l3 8615	Glutathione biosynthesis; Oxidoreductase activity	58, Down	31.64	0.02
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hmgcr	m29249	Cholesterol biosynthesis; lipid metabolism; oxidoreductase	64, Down	31.65	0.03
Fibronectin 1	Fn1	x15906	Oxidoreductase activity; heparin binding	68, Down	31.65	0.07
Prolyl 4-hydroxylase alpha subunit	P4ha1	x78949	Oxidoreductase activity; Protein metabolism	68, Down	31.65	0.02
<i>2d: Genes associated with stress response</i>						
Coagulation factor 2	F2	x52835	Acute-phase response; Blood coagulation; Proteolysis and Peptidolysis; Trypsin activity	158, Up	21.53	0.06
Bile acid-Coenzyme A: amino acid N-acyltransferase	Baat	d43964	Acute-phase response; Bile acid metabolism	178, Up	19.38	0.03

Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
Bile acid CoA ligase	LOC79111	af242189	Acute-phase response; Bile acid metabolism	198, Up	27.68	0.01
Protein associating with small stress protein PASS1	Pass1	af168362	Stress associated protein	69, Down	31.65	0.02
Crystallin, alpha B	Cryab	M 55534	Heat shock protein activity	44, Down	31.64	0.05
Serine (or cysteine) proteinase inhibitor, clade H, member 1	Serpinh1	M 69246	Chaperone activity; heat shock protein activity	65, Down	31.65	0.02
<i>2e: Genes associated with immune function</i>						
Tubulin, beta 5	Tubb5	ab011679	Natural killer cell mediated cytolysis; MHC class I protein binding	58, Down	31.64	0.04
Scavenger receptor class B, member 1	Scarb1	af071495	Immune function	69, Down	31.65	0.01
Tubulin tyrosine ligase	Ttl	af207605	Tubulin-tyrosine ligase activity; Immune function	62, Down	31.65	0.05
Interferon, beta 1	Ifnb1	d87919	Cytokine activity; defense response	65, Down	31.65	0.06
Interferon-related developmental regulator 1	Ifrd1	j0 4511	Neurogenesis; Immune function	64, Down	31.64	0.01
Interleukin 1 receptor accessory protein	Il1rap	u48592	Interleukin-1 receptor activity	69, Down	31.65	0.01
<i>2f: Genes associated with cell integrity and transportation</i>						
Ribosomal protein S16	Rps16	x17665	RNA binding; Protein biosynthesis; Structural constituent of ribosome	196, Up	19.37	0.04
Solute carrier family 10,member 1	Slc10a1	m77479	Organic anion transport; sodium ion transport	155, Up	21.53	0.04
Ferritin, heavy polypeptide 1	Fth1	u58829	Ferric iron binding and transport	183, Up	21.53	0.01
Syndecan 2	Sdc2	m81687	Cytoskeletal protein binding; integral to membrane	152, Up	27.68	0.05
Ribosomal protein L13A	Rpl13a	x68282	Protein biosynthesis; structural constituent of ribosome	201, Up	9.68	0.01
ER transmembrane protein Dri 42	Dri42	y07783	Protein transportation activity	162, Up	31.65	0.03

Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
Regulator of G-protein signaling 5	Rgs5	af241259	Signal transducer activity; signal transduction	72, Down	31.65	0.05
Tyrosine3-monooxygenase /tryptophan-5-monooxygenase activation protein	Ywhah	d17445	Intracellular protein transport; protein domain specific binding	72, Down	31.64	0.04
Proteasome (prosome, macropain) subunit, alpha type 1	Psm1	d90265	Hydrolase activity; transporter activity; ubiquitin-dependent protein catabolism	70, Down	31.65	0.06
Syntaxin 5a	Stx5a	l2 0822	Intracellular protein transport	58, Down	31.65	0.03
Syntaxin binding protein Munc18-2	Stxbp2	u20283	Intracellular protein transport; protein secretion	67, Down	31.65	0.06
ADP-ribosyltransferase 1	Adprt	u94340	DNA bindingtransferring glycosyl groups; transferase activity; Transfer pentosyl groups	57, Down	31.64	0.04
Reversion induced LIM gene	Ril	x76454	Intracellular signaling cascade	66, Down	32.39	0.06
Catechol-O-methyltransferase	Comt	z12651	S-adenosylmethionine-dependent methyltransferase activity; neurotransmitter catabolism; transferase act.	57, Down	31.65	0.01
Similar to ruvB-like protein 1	Ruvb1	ab001581	Enhance plasminogen activation	65, Down	31.64	0.05
Agrin	Agrn	m64780	Plasma membrane organization and biogenesis	66, Down	31.65	0.04
Reticulon 1	Rtn1	u17603	Integral to membrane; molecular function unknown	64, Down	31.65	0.03
Lamina-associated polypeptide 1C	Lap1c	u19614	Lamin binding	63, Down	31.65	0.05
lamin B1	Lmb1	u72353	Intermediate filament; kinesin complex	69, Down	31.65	0.05
Proteasome (prosome, macropain) 26S subunit, ATPase 3	Psmc3	u77918	Mitochondrial inner membrane; nucleotide binding	57, Down	31.64	0.02
Telomerase associated protein 1	Tep1	u89282	Cell division	59, Down	31.65	0.01
Dynactin 1	Dctn1	x62160	Cytoplasmic dynein complex; kinesin complex; microtubule motor activity	69, Down	32.39	0.04
Three-PDZ containing protein similar to C. elegans PAR3	Par3	ab005549	Establishment and/or maintenance of epithelial cell polarity; tight junction	68, Down	31.65	0.06
Sodium-dependent dicarboxylate transporter	Slc13a3	af081825	Ion transport	62, Down	31.64	0.02

Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
<i>2g: Genes associated with other cellular processes</i>						
D-amino acid oxidase	Dao1	ab003400	D-amino-acid oxidase activity	58, Down	31.65	0.06
Drebrin-like	Dbn1	ab038364	Actin binding; dendrite	65, Down	31.64	0.01
RNA binding protein p45AUF1	Hnrpd	ab046618	Regulates cytosolic mRNA degradation	71, Down	31.65	0.04
Inhibitor of DNA binding 3	Id3	af000942	Inhibit transcription	67, Down	31.64	0.05
Serine/threonine protein kinase	LOC286993	af084205	Protein serine/threonine kinase activity	70, Down	31.65	0.07
Zuotin related factor 2	Zrf2	af118853	Negative regulation of cell growth	45, Down	31.64	0.03
Serum-inducible kinase	Snk	af136583	Cell division	68, Down	31.65	0.04
Splicing factor YT521-B	YT521	af144731	Nuclear mRNA splicing	61, Down	31.64	0.05
P34 protein	P34	af178669	Cell division	52, Down	31.65	0.04
Protein kinase	Prkwnk1	af227741	Protein phosphorylation/dephosphorylation	68, Down	31.64	0.05
Zinc finger	Zdhhc2	af228917	DNA binding motif; Transcriptional regulation	51, Down	31.65	0.04
Asialoglycoprotein receptor 2	Asgr2	af230645	Endocytosis; heterophilic cell adhesion; sugar binding	71, Down	31.65	0.07
Serine-arginine-rich splicing regulatory protein 86	Srrp86	af234765	mRNA splicing activity	74, Down	31.64	0.05
Brain-enriched SH3-domain protein Besh3	Besh3	af254801	Brain-enriched SH3-domain protein	65, Down	31.64	0.02
Myosin IX A	Myo9a	aj001713	Binds with ATP; ATPase activity	63, Down	31.65	0.04
Ornithine decarboxylase antizyme 1	Oaz1	d10706	Ornithine decarboxylase inhibitor activity	52, Down	31.65	0.07
Hematopoietically expressed homeobox	Hhex	d86383	Cell differentiation; general transcriptional repressor activity; hemopoiesis	67, Down	31.65	0.02

Table 4.2 (continued)

Gene Name	Symbol	Genbank #	Gene Function	Percent Change ^b	FDR, (%) ^c	p Value
Myosin heavy chain, polypeptide 6						
	Myh6	j 00751	ATP binding; actin binding; calmodulin binding; cytoskeleton; muscle development; myosin	58, Down	31.64	0.03
Protein phosphatase 1A, magnesium dependent, alpha isoform	Ppm1a	j0 4503	Catalytic activity; phosphoprotein phosphatase activity; protein amino acid dephosphorylation	58, Down	31.64	0.03
Retinol binding protein 1	Rbp1	m16459	Retinoid binding and metabolism	70, Down	31.65	0.07
Prosaposin	Psap	m19936	Lysosome	74, Down	31.64	0.07
Aldehyde dehydrogenase family 1, subfamily A4	Aldh1a4	m23995	Bile acid biosynthesis; Fatty acid metabolism	69, Down	31.65	0.05
Deoxyuridinetriphosphatase (dUTPase)	Dut	u64030	dUTP metabolism; hydrolase activity	66, Down	31.65	0.02
Progesterin induced protein	dd5	x64411	RNA binding; protein ubiquitination	54, Down	31.64	0.05
Sperm outer dense fiber major protein 2	Odf2	x95272	Outer dense fiber; spermatid development	66, Down	31.65	0.01

^aE+ = Infected fescue seed diet

^bPercent Change = Regulation of the gene in E+ compared to E- rats with Up indicating Upregulated genes and Down representing Down-regulated genes. The values represent percent changes by which genes are differentially expressed.

^cFDR,% = Percentage of false discovery rate

CHAPTER V

EFFECT OF ERGOT ALKALOIDS ASSOCIATED WITH FESCUE TOXICOSIS ON HEPATIC CYTOCHROME P450 AND ANTIOXIDANT PROTEINS

1. ABSTRACT

Intake of ergot alkaloids found in endophyte-infected tall fescue grass is associated with decreased feed intake, and reduction in body weight (BW) gain. The liver is one of the target organs of fescue toxicosis with up-regulation of genes involved in xenobiotic metabolism and down-regulation of genes associated with antioxidant pathways. It was hypothesized that short-term exposure of rats to ergot alkaloids would change hepatic cytochrome P450 (CYP) and antioxidant expression, as well as reduce antioxidant enzyme activity and hepatocellular proliferation rates. Hepatic gene expression of various CYPs, selected nuclear receptors associated with the CYP induction, and antioxidant enzymes were measured using real-time PCR. Hepatic expression of CYP, antioxidant and proliferating cell nuclear antigen (PCNA) proteins were measured using western blots. The CYP3A1 protein expression was evaluated using primary rat hepatocellular cultures treated with ergovaline, one of the major ergot

alkaloids produced by fescue endophyte, in order to assess the direct role of ergot alkaloids on CYP induction. The enzyme activities of selected antioxidants were assayed spectrophotometrically. While hepatic CYP and nuclear receptor expression were increased in ergot alkaloid-exposed rats, the expression and activity of antioxidant enzymes were reduced. This could potentially lead to increased oxidative stress, which might be responsible for the decrease in hepatocellular proliferation after ergot alkaloid exposure. This study demonstrated that even short-term exposure to ergot alkaloids can potentially induce hepatic oxidative stress which can contribute to the pathogenesis of fescue toxicosis.

2. INTRODUCTION

Exposure to ergot alkaloids in endophyte (*Neotyphodium coenophialum*)-infected tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh., formerly *Festuca arundinacea* (Schreb.)] is associated with various pathophysiological problems in both ruminant and nonruminant animals which are collectively referred to as fescue toxicosis (Schoning et al., 2001, Settivari et al., 2006). Over half of the tall fescue in the United States is infected with endophyte, resulting in more than \$600 million in losses per year to the beef cattle industry (Hoveland, 1993). The clinical signs of fescue toxicosis include decreased feed intake, body weight (BW) gain, conception rate, milk production, and offspring survival, as well as increased abortion rate, placenta size, peripheral vasoconstriction, rough hair coat, salivation, and hyperthermia during heat stress (Cross et al., 1995; Strickland et al., 1993). However, there are significant data gaps regarding the underlying

cellular and metabolic aberrations associated with exposure to ergot alkaloids which limit the development of pharmacological strategies to reduce these problems (Settivari et al., 2006).

Xenobiotic-metabolizing enzymes play a central role in the metabolism, elimination and detoxification of drugs and foreign compounds introduced into the body (Xu and Kong, 2006). These enzymes are present in abundance at either a basal unstimulated level and/or are inducible after exposure to xenobiotics (Xu and Kong, 2006). Cytochrome P450 (CYP) enzymes are associated with several biological interactions involving hydroxylation, epoxidation, dehydrogenation, and nitrogen dealkylation, and oxidative deamination (Bhaskarannair et al., 2006). Although CYP-mediated reactions are essential for xenobiotic detoxification, they can also generate reactive oxygen species (ROS) (Bhaskarannair et al., 2006). All aerobic cells contain a battery of defenses to prevent cellular oxidative damage associated with ROS and/or active metabolites generated by CYPs, especially when there are low cellular concentrations of antioxidant enzymes (Tiedge et al., 1998).

While most of the tissues and organs in the body are well equipped with CYPs and antioxidant enzymes (Xu and Kong, 2006), the liver is the most important organ for xenobiotic metabolism and detoxification and has been identified as one of the target organs of fescue toxicosis in cattle (Oliver, 1997; 2005). Piper et al. (1991) noted that liver-specific enzymes increased in animals following long-term exposure to endophyte-infected fescue, and a component of the fescue toxicosis response in rats is a decrease in liver weight (Settivari et al., 2006).

Although not completely understood, interactions have previously been reported between ergot alkaloids and CYPs (Moubarak et al., 2000; 2003b), as well as antioxidants (Lakritz et al., 2002); and differentially-expressed genes in the livers of rats fed ergot alkaloids include up-regulation of those involved in xenobiotic metabolism and down-regulation of genes associated with antioxidant pathways (Settivari et al., 2006). The primary objective of this study was to determine the overall impact of ergot alkaloids on hepatic CYP and antioxidant proteins and to demonstrate hepatocellular damage induced by those effects. In addition, CYP3A1 protein expression was evaluated in primary hepatocellular cultures treated with ergovaline (EV), one of the major ergot alkaloids produced by fescue endophyte (Garner et al., 1993), in order to assess the direct effect on CYP induction. It was hypothesized that short-term intake of ergot alkaloids by rats kept at thermoneutrality would change hepatic CYP and antioxidant expression, as well as reduce antioxidant enzyme activity and hepatocellular proliferation rates.

3. MATERIALS AND METHODS

Animals and treatment diets

Sprague-Dawley male rats ($n = 12$ per treatment group; 42 days of age; 185 to 215 g BW; Charles River Laboratories, Willington, MA), were maintained at 21°C ambient temperature and $50 \pm 5\%$ relative humidity as previously reported (Spiers et al., 2005a). Throughout the study, feed and water were provided *ad libitum*. Composition of control and treatment diets were as previously described, with the ergot alkaloid-containing diet (E+) adjusted to provide a specific amount (91.5 µg/kg BW/day) of EV,

which is known to produce clinical signs of fescue toxicosis in rats (Spiers et al., 2005a). The E+ diet contained 4,100 ppb of EV, whereas the control (E-) contained a concentration of 22 ppb on dry matter basis as measured by HPLC (Rottinghaus et al., 1993). All procedures on animals followed guidelines for humane treatment set by the University of Missouri Animal Care and Use Committee.

Treatment period and sample collection

The treatment schedule consisted of an eight-day recovery period after surgery to implant telemetric transmitters ip, followed by a five-day pretreatment period and five days of exposure to either E+ or E- diets. After the five days of exposure, rats were euthanized using ketamine (87 mg/ml) and xylazine (13 mg/ml) at 0.1 mg/100 gm BW, administered im, followed by blood collection via intracardiac puncture. As reported previously (Settivari et al., 2006), selected visceral organs (i.e., liver, adrenal, kidney, heart, and testes) were collected from each animal following euthanasia and weighed. A transverse section from the middle lobe of the liver (approximately 5 g) was fixed in 10% neutral buffered formalin for histological examination. Another portion (approximately 5 g) was placed in RNase-free Eppendorf tubes (Eppendorf Biopur, Westbury, NY) and snap-frozen in liquid nitrogen for real-time PCR (RT-PCR).

RNA extraction and cDNA conversion

Total RNA was extracted from liver samples using RNeasy Midi Kit (Qiagen Inc., Valencia, CA) and purified using rDNase-1 (Ambion Inc., Austin, TX). The rDNase-1 enzyme in the RNA sample was inactivated using heat (70°C) and a phenol, chloroform, isoamyl alcohol solution (25:24:1). Purified total RNA was concentrated using Microcon filters (Amicon Bioseparations, Bedford, MA), following manufacturer's instructions.

The RNA concentrations were measured using a ND–1000 spectrophotometer (Nanodrop Technology, Wilmington, DE). Concentrated total RNA (15 µg) from rats fed E+ or E- diet was reverse – transcribed to cDNA using oligo dT (IDT DNA, Coralville, IA), random hexamers (IDT DNA, Coralville, IA) and reverse transcriptase (Stratagene, La Jolla, CA). The cDNA obtained, was purified using Microcon YM30 filters (Millipore Corp., Bedford, MA).

Quantitative RT - PCR

Quantitative RT – PCR assays were performed to measure the relative hepatic expression of mRNA representing various CYP isoforms, selected nuclear receptors associated with the CYP induction, and antioxidant enzymes. The coding sequence and chromosome contiguous sequence for each gene were acquired through the NCBI public database (www.ncbi.nlm.nih.gov) and the exon-exon junctions were determined using BLAT Search Genome (Kent, 2002). Gene-specific primers were designed with Primer Express program (Applied Biosystems, Warrington, UK) using default settings (primer T_m = 58 to 60°C, GC content = 30 to 80%, length = 9 to 40 nucleotides). The primers were designed to be exon-spanning to avoid amplification of contaminating genomic DNA (Tables 5. 1 and 5. 2). Glyceraldehydes -3- phosphate dehydrogenase (GAPDH) was used as the house keeping gene and the PCR results were normalized to rat GAPDH expression. The annealing temperature for the primers was standardized using gradient PCR and 3% agar gels. The reaction mixture consisted of 2X SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK), 50 nM forward and reverse primers and 0.1 ng/µl cDNA. Thermal cycling was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems Warrington, UK) under factory default conditions

(50°C, 2 min; 95°C 10 min; and 40 cycles at 95°C, 15 sec; 60°C, 1 min). Each gene was measured in triplicates and the formation of a single PCR product was confirmed using dissociation curves. Negative controls, which consisted of all of the components of PCR mix except cDNA, were used for all the primers. The normalized C_T (the cycle number at which the fluorescence passes the threshold) values for each gene in all the 12 animals of each group were averaged, and the relative fold-change in the expression of the gene in animals fed E+ versus E- diet was calculated. Statistical analysis of the data was performed by comparing the C_T values of E+TN and E+HS rats for each gene using one-way analysis of variance.

Primary hepatocellular cultures

Rat hepatocytes were isolated by a previously described collagenase-perfusion protocol (Seglen, 1976). The abdomens of anesthetized rats were opened, and the portal veins were cannulated. The inferior vena cava of each rat was ligated above the level of the renal veins to prevent the perfusion to kidneys. The chest was opened, and the right atrium portion was cannulated. To remove the blood, each rat's liver was first perfused with Krebs-Ringer-bicarbonate buffer (KRB) containing 0.25 mM EGTA for 1 to 2 min and then with KRB containing 2 mM $CaCl_2$ and collagenase (35 mg/100 ml) for 10 to 15 min. The liver was then removed from each rat and transferred to a beaker containing 10 to 15 ml KRB buffer medium. The liver tissue was minced to disperse cells using scissors. The cell suspension was filtered through a nylon mesh and centrifuged at 50 X g for 30 s at 24°C. Isolated hepatocytes were washed twice with 50 ml KRB buffer containing 0.5% bovine serum albumin and 15 mM glucose by centrifugation as above and resuspended in the same buffer. Cell viability, as assessed by the exclusion of trypan

blue, was $85 \pm 5\%$. The hepatocytes (3×10^6 cells/60 mm dish) were plated on to collagen-coated culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. After 24 hours of incubation at 37°C in a 5% CO_2 incubator, the medium was changed to DMEM containing 0.1% fetal bovine serum with or without EV (i.e., 0, 1, 10, or 100 nM). After an additional 24 hours of incubation at 37°C in DMEM with or without EV, cells were treated with trypsin and collagenase for 5 min, followed by neutralization with medium. After centrifugation of the cell suspension at 1,500 rpm for 5 min. and removal of the supernatant, the pellet was stored at -80°C until immunoblots were performed.

Immunoblots

Protein levels of CYP3A1 and selected antioxidants were evaluated in liver samples collected from rats exposed to either E+ or E- diet using standard immunoblot protocols described by Bowles et al. (2004). Liver samples were minced thoroughly in 200 μl of ice-cold buffer (50 mM Tris-HCl; pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β -mercaptoethanol, 1 nM PMSF, 2 nM leupeptin, 1 nM pepstatin A, 10% vol/vol glycerol) and subjected to 4 X 30 s homogenizations at a speed of 4.0 to 5.0 using a FASTPREP rapid homogenizing system (QBiogene, Carlsbad, CA) with samples cooled on ice for 5 min between pulses. For primary hepatocellular cultures, cells were thawed and homogenized as described above. Whole cell homogenates were centrifuged at 500 g for 5 min and pellets containing debris were discarded. With respect to PCNA protein, nuclear extracts were prepared from the livers of rats exposed to either E+ or E- diet following the manufacturer's protocol (Nuclear Extraction Kit, Active Motif, Carlsbad, CA). These extracts were later used to determine the amount PCNA protein as a marker

for hepatocellular proliferation. Protein concentrations for all fractions were determined using a Bradford protein assay kit (BioRad). Thirty micrograms of protein was electrophoresed on 4 to 15% SDS polyacrylamide gels and transferred to nitrocellulose membrane at 100 V for 1 h at 4°C. Following transfer, nitrocellulose membranes were blocked for 1 h in Tris-buffered saline solution containing 5% dry milk, 0.1% Tween-20 and probed with rabbit polyclonal antibodies (Abcam Inc., Cambridge, MA) overnight at 4°C as follows: CYP3A1 (1:1000; Ab3572), GPx (1:1000; Ab16798), SOD (1:1000; Ab13498), Catalase (1:500; Ab16731) and PCNA (1:500; Ab2426). The primary antibody used to detect hepatic CYP3A1 was specific for human CYP3A4 and had cross reactivity with rat CYP3A1. Nitrocellulose membranes were then washed and incubated in horseradish peroxidase-labeled secondary antibody for 1 h. Enhanced chemiluminescence techniques were used to visualize the immunoblots following manufacturer's protocol (ECL, Amersham, Piscataway, NJ). Digitized images of the luminograms were used for densitometric measurements with Scion Image software (NIH, Bethesda, MD) with care taken to prevent saturation

Immunohistochemical staining for CYP3A1 in histological sections of rat livers

Protein expression of CYP3A1 was visualized in the liver of rats exposed to E+ or E- diet using a standard immunohistochemical staining. Liver samples were fixed in neutral buffered 10% formalin for a minimum of 24 hours, embedded in paraffin and sectioned serially at 4 µm thickness. Sections were floated onto positively charged slides (Thomas Sci, Swedesboro, NJ), deparaffinized and steamed in citrate buffer at pH 6.0 for 30 min to achieve antigen retrieval. Sections were then incubated with avidin-biotin two-step blocking solution to inhibit background staining and in 3% H₂O₂ to inhibit

endogenous peroxidase. The sections were incubated overnight at 4°C with a 1: 500 dilution of primary rabbit monoclonal antibody to CYP3A1 (Abcam Inc., Cambridge, MA), which has cross-reactivity with rat CYP3A1. Following the incubation, sections were incubated with biotinylated anti-rabbit secondary antibody in PBS containing 15 mM sodium azide and peroxidase-labeled streptavidin (Dako LSAB+ kit, peroxidase, K0690). Images were captured at 400X magnification (Insight Color Camera Sterling Heights, MI).

Antioxidant activity assays

Rat liver samples from both treatment groups were homogenized as described earlier and total protein concentrations were determined using a Bradford protein assay kit (BioRad). The enzyme activities of superoxide dismutases (SODs), catalase and glutathione peroxidase (GPx) were assayed spectrophotometrically following manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). The specific activity of hepatic GPx, SODs and catalase was expressed as unit μg^{-1} liver total protein. Glutathione peroxidase activity was measured indirectly by a coupled reaction with glutathione reductase. Glutathione peroxidase reduces hydroperoxides and results in the production of oxidized glutathione, which in turn is recycled to its reduced state by glutathione reductase and NADPH. Glutathione reductase oxidizes NADPH to NADP^+ , and it is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample. Hepatic SOD activity was assayed using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The enzyme activity (1 unit) was measured as the

amount of enzyme required to exhibit 50% dismutation of superoxide radicals. Hepatic catalase activity was assayed using its peroxidatic function using methanol and H₂O₂.

Statistical analyses

Mean values for core temperature, feed intake and body weight were computed as a function of time and treatment group. These parameters were analyzed by repeated measures ANOVA (Mixed procedure) of SAS (SAS Inst., Inc., Cary, NC) using treatment, time and animal as class variables to determine overall treatment and time-related differences, and treatment x time interactions. As previously mentioned with respect to the quantitative RT-PCR analyses, C_T values of both groups were analyzed using one way analysis of variance and proc univariate analysis of SAS to compare the mean values for the E+ and E- rats. Similarly, enzyme activities and densitometric values for various parameters were analyzed for significance using one-way analysis of variance and proc univariate analysis of SAS. Mean for CYP3A1 protein expression in dose response experiment was analyzed as a function of EV concentration. The above parameter was analyzed by standard least square model fit (JMP[®], SAS Institute Inc., Cary, NC). If a significant difference existed for any comparison, then a Tukey - Kramer multiple comparisons test (Steele and Torrie, 1980) was performed ($\alpha = 0.05$) to determine specific effects. All data is reported as mean \pm standard error of mean. A p-value less than 0.05 were considered significant between E- and E+ rats.

4. RESULTS

Effect on physiological and pathological parameters

Rats fed an E+ diet significantly ($p < 0.05$) reduced feed intake, body weight gain, and core body temperature, as compared to the rats fed the E- diet. Concentrations ($p < 0.05$) of serum prolactin, glucose, cholesterol and urea nitrogen were also lower ($p < 0.05$) in rats fed E+ versus E- diet at the end of five-day treatment. These results, as well as the absence of hepatic histopathological abnormalities other than the loss of lipid and glycogen droplets in the livers of rats fed E+ diet, were reported previously by Settivari et al. (2006).

Effects on hepatic CYP isoforms, PXR and RXR

While a significant increase in gene expression was observed for various CYP isoforms, such as CYP1A1 ($p = 0.02$), CYP2C9 ($p = 0.03$), CYP2E1 ($p = 0.02$) and CYP3A1 ($p = 0.01$) in the livers of rats fed E+ versus E- diet, rats fed the E+ diet showed a significant reduction in hepatic gene expression for CYP3A7 (Figure 5. 1). Hepatic gene expression of CYP2C13 ($p = 0.20$), CYP1A2 ($p = 0.41$) and CYP2C6 ($p = 0.13$) were not different between rats fed E+ and E- diets. Genes for the CYP-inducing nuclear receptors pregnane X receptor (PXR; $p = 0.02$) and retinoid X receptor (RXR; $p < 0.01$) were upregulated in the livers of rats fed E+ diet, compared to rats fed the E- diet (Figure 5. 1). At the end of five-day treatment period, protein expression of the major CYP isoform, CYP3A1, was induced ($p < 0.01$) in the livers of E+-treated rats compared to the livers of rats fed the E- diet (Figure 5. 2), and fixed liver sections from rats fed the E+

diet stained more intensely for this isoform than rats fed the E- diet (Figure 5. 3).

Primary hepatocellular cultures from healthy rats exhibited an EV dose-dependent induction of CYP3A1 protein expression compared to hepatocytes not exposed to EV (Figure 5. 4).

Effects on hepatic antioxidant enzymes PCNA

Hepatic gene expression of two SODs (CuZn-SOD; $p = 0.01$) and Mn SOD; $p = 0.05$), catalase ($p = 0.05$) and GPx ($p = 0.02$) were significantly down-regulated, while the hepatic expression of phosphohydroxyl glutathione peroxidase (PH-GPx; $p = 0.01$), the monomeric isoform of GPx, was up-regulated significantly in the livers of rats fed E+ diet compared to E- rats (Figure 5. 5). Hepatic gene expression of glutathione-S-transferase (GST; $p = 0.50$), heme oxygenase (HO; $p = 0.41$) and nitric oxide synthase ($p = 0.84$) did not differ between E+ and E- groups. At the end of treatment, protein expression (Figure 5. 6) and antioxidant enzymatic activity (Figure 5. 7) of three major antioxidants, CuZn-SOD, catalase and GPx, were lower ($p < 0.05$) in livers of rats fed E+ diet than in E- rats. Protein expression of PCNA was lower ($p = 0.02$) in E+ compared to E- rat livers by the end of treatment period (Figure 5. 8).

5. DISCUSSION

The liver has been discussed as a possible target organ in fescue toxicosis (Oliver, 1997; 2005; Piper et al, 1991), and ergot-alkaloid-induced alterations in hepatic gene expression in several important physiological pathways, including energy metabolism, growth and development, as well as xenobiotic metabolism and antioxidant activity, have

been previously reported (Settivari et al., 2006). The results reported in this manuscript demonstrate the overall impact of fescue toxicosis on hepatic CYP and antioxidant protein expression and suggest a possible role for these effects in the pathogenesis of fescue toxicosis in livestock species (Figure 5. 9).

Effects on hepatic CYP isoform expression and oxidative stress

As hypothesized, short-term intake of ergot alkaloids by rats kept at thermoneutrality resulted in changes in hepatic CYP expression, which could potentially be related to the role of oxidative stress in the pathogenesis of fescue toxicosis (Figure 5. 9). The overall up-regulation of CYP enzyme expression in rats fed a diet containing ergot alkaloids in the present study is consistent with the results of earlier experiments in sheep (Zanzalari et al., 1989), rats and cattle (Thompson et al., 2001).

Ergot alkaloids produced by various fungal organisms are known to be substrates for members of the CYP3A subfamily (Zuber et al., 2002). It is likely that the ergot alkaloid-induced overexpression of hepatic CYP3A1 (orthologous isoform of human CYP3A4 in rats; Murray and Correia, 2001; Zuber et al., 2002) gene and protein in the present study facilitate the metabolism of various ergot alkaloids associated with fescue toxicosis. Interactions between ergot alkaloids and the CYP3A enzyme have been elucidated previously, and concomitant administration of ergot alkaloids and CYP3A inhibitors has been reported to predispose susceptible individuals to the development of ergotism (Dresser et al., 2000). Moubarak et al. (2000) observed hydroxylation of the ergot alkaloid ergotamine by CYP3A enzyme present in beef liver microsomes, and, in another study, Moubarak et al. (2003b) induced CYP3A enzyme levels in rat liver microsomes and then treated the preparation with ergotamine, resulting in greater

metabolism of ergotamine and production of dihydroxy derivatives than in controls due to CYP3A-induced microsomes. Peyronneau et al. (1994) reported metabolism of bromocriptine, an ergot alkaloid similar to ergotamine, by CYP3A subfamily in rat liver microsomes treated with dexamethasone, a CYP3A inducer, and Althus et al. (2000) observed the metabolism of another ergot alkaloid, alpha-dihydroergocryptine, by CYP3A isoforms. Interestingly enough, the gene for CYP3A7, which has a specific role in 16 alpha-hydroxylation of steroids (Daly, 2006) and the bioactivation of several pro-mutagens, including aflatoxin B1 (Li et al., 1997), was down-regulated in the livers of E+ rats in the present study. While the role of this member of the CYP3A subfamily in the metabolism of ergot alkaloids has yet to be elucidated, it is possible that the observed alteration in this gene is similar to the effect of E+ diet in mice, where hepatic expression of enzymes involved in steroid metabolism was decreased (Bhusari et al., 2006).

Like the CYP3A subfamily, the CYP2C enzymes are abundant in rat liver. Of the three CYP2C isoforms tested in this study, two (CYP2C9, CYP2C13) appeared to be up-regulated in E+ fed rats, with only CYP2C9 expression reaching statistically significant level (Figure 5. 1). The CYP2C isoforms are associated with oxidation of the mycotoxin aflatoxin B1 as well as dihydropyridines, and these enzymes also hydroxylate various steroids (Zuber et al., 2002). It is possible that CYP2C isoforms may oxidize ergot alkaloids, as well as aflatoxins.

Rats fed an E+ diet in the present study increased CYP2E1 gene expression in hepatic tissue. Overexpression of CYP2E1 has been shown to induce chronic oxidative stress by generating more ROS, possibly leading to hepatocellular injury and death (Schattenberg et al., 2004). Hepatocytes containing over-expressed CYP2E1 have been

reported to release diffusible mediators which activate hepatic stellate cells and alpha smooth muscle actin, as well as intracellular and secreted collagen type 1 protein, intra- and extracellular hydrogen peroxide, and lipid peroxidation products (Nieto et al., 2002).

Intake of an E+ diet in the present study increased hepatic gene expression of CYP1A1 with no change in CYP1A2. Along with UDP-glucuronosyl transferase, CYP1A1 plays a crucial role in the metabolism of xenobiotic compounds (Nishizawa et al., 2005). It is known that CYP1A1, 1A2 and 3A4 isoforms bioactivate certain endo and xenobiotics (Bhaskarannair et al., 2006; Haas et al., 2006). These isoforms convert estrogens into quinones and semiquinones resulting in oxidative damage to DNA and to lipids (Gallicchio et al., 2006). It is evident from the results of the present study that transcriptional activation of CYP1A1, as well other CYP isoforms, in response to ergot alkaloids has the potential to increase oxidative stress.

Direct versus indirect effects on hepatic CYP isoform expression

While, in all likelihood, the overall impact of fescue toxicosis on hepatic CYP proteins involves both direct and indirect effects of ergot alkaloids, the results of the present study helped to elucidate the direct effects of ergot alkaloids on hepatic CYP expression from indirect effects of endophytic toxins related to decreased nutrient and caloric intake and/or utilization. The dose-dependent induction of CYP3A1 protein expression in primary hepatocellular cultures in response to ergovaline, one of the primary ergot alkaloids associated with fescue toxicosis, clearly demonstrated the ability of ergopeptine alkaloids to directly induce various CYP isoforms independent of reduced nutrient and caloric intake. Based on the differential expression of hepatic CYP isoforms in starved mice which was attributed by Bauer et al. (2004) to reduced feed intake, it is

likely that the overexpression of CYP isoforms and potential production of ROS observed in the present study were also due, in part, to reduced nutrient and caloric intake.

Effects on hepatic gene expression of selected CYP-inducing nuclear receptors

Fescue toxicosis induced the up-regulation of selected hepatic nuclear receptor genes PXR and RXR. Both these nuclear receptors are ligand-activated by a large number of structurally and physiologically diverse endogenous ligands (e.g., steroid hormones, vitamins and, bile acids), as well as a wide range of xenobiotics, such as rifampin, protease inhibitors and environmental contaminants (Lamba et al., 2004). Pregnane X receptor is an important component of the body's defense mechanism against toxic substances, and it serves as a generalized sensor for exposure to hydrophobic xenobiotics (Kliewer et al., 2002). The activation of PXR results in the induction of an elaborate network of genes involved in all aspects of xenobiotic metabolism and detoxification (Kliewer, 2003), and PXR, which is primarily expressed in liver, small intestine and colon, induces multiple CYP isoforms (Bertilsson et al., 1998; Pascussi et al., 2000). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor and transactivates ER6 (everted repeat with a 6 bp spacer) elements upstream of the CYP genes (Waxman, 1999). This transactivation is followed by recruitment of co-activator protein, SRC-1 (steroid receptor coactivator-1), leading to transcriptional activation of genes, which code for CYPs (Savas et al., 1999). Therefore the up-regulation of these nuclear receptors provides a mechanism for the induction of various CYP isoforms in response to either endophyte toxins themselves and/or, due to reduced feed intake (Bauer et al., 2004).

Effect on hepatic antioxidant enzyme expression and activity and oxidative stress

Short-term intake of ergot alkaloids in the present study changed hepatic antioxidant enzyme expression and activity, and it was potentially related to the role of oxidative stress in the pathogenesis of fescue toxicosis (Figure 5. 9). The possible consequences of increased CYP-associated xenobiotic metabolism include the production of reactive molecules, which may be more toxic than the parent xenobiotic, as well as oxygen free radicals (Liska, 1998; Percival, 1997). Antioxidant enzymes can inhibit free radical production by chelating the transition metal catalysts, breaking chain reactions, reducing concentrations of ROS, and scavenging initiating radicals (Liska, 1998; Scandalios, 1997). The decreased hepatic expression and activity of antioxidant enzymes, as observed in the study, have the potential to predispose animals to hepatic insult induced by oxidative stress (Tiedge et al., 1998)

This is the first report, to our knowledge, of the effect of ergot alkaloids on hepatic expression and activity of SOD, which catalyses the conversion of superoxide anions into H₂O₂ and is one of the primary enzymatic defenses against ROS. Mammalian cells have a SOD enzyme containing manganese at its active site in mitochondria and one with copper and zinc at the active site in the cytosol (Scandalios, 1997). Minor changes in Mn-SOD expression can have a significant impact on the antioxidant status of mitochondria, since a 50% reduction in its expression results in a functional decline of oxidative phosphorylation, an increase in oxidative stress, and an increase in apoptosis rate (Wheeler et al., 2001). In the present study, hepatic gene expression of both forms of

SOD was down-regulated in rats fed an E+ diet. This down-regulation in SOD gene expression, in conjunction with diminished activity, could potentially increase superoxide anions within the mitochondria and cytoplasm. Likewise, there could be impairment of cellular function with increased oxidative stress that is possibly related to superoxide radical-induced inhibition of catalase activity (Marczuk-Krynicka et al., 2003).

Intake of an E+ diet in the present study also reduced gene and protein expression and activity of GPx and catalase. Catalase is an important antioxidant enzyme located within the peroxisomes and GPx is located in the cytosol (Liska, 1998). These enzymes are involved in the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, into water and molecular oxygen (Liska, 1998). It is anticipated that cells containing low levels of one or more of these enzymes would be particularly vulnerable to oxidative damage (Liska, 1998; Tiedge et al., 1998), and this appears to be the case with the rats fed the E+ diet in the present study. Reductions in catalase and GPx antioxidant enzyme expression and activity in E+ rats would impair conversion of hydrogen peroxide to water and resulting in persistence of the hydroxyl radical within hepatocytes. The resulting hydroxyl radicals cause site-specific damage on the inner mitochondrial membrane and critical cellular components leading to cellular function impairment and apoptosis (Tiedge et al., 1998).

While most of the genes for antioxidant enzymes evaluated in this experiment were down-regulated following exposure to ergot alkaloids, the gene for PH-GPx was actually up-regulated. The selenium-dependent enzyme PH-GPx detoxifies hydroperoxides by reducing them to alcohols (Godeas et al., 1994). While the basis for the up-regulation of this gene in the present study is unknown, the increased expression

of this gene as an adaptive mechanism to increased peroxide concentration within hepatocytes cannot be ruled out. It is also possible that there is some relationship between the up-regulation of this gene and the observed down-regulation of the hepatic gene expression, as well as enzymatic activity, of GPx. Reduced antioxidant activities of E+ rats in the present study are consistent with earlier reports. Lakritz et al. (2002) reported reduced blood glutathione levels in cattle fed ergot-alkaloid containing diet during heat stress. Gorini et al. (1988) observed a reduction in forebrain glutathione levels in male rats after chronic i.p. treatment with dihydroergocristine and dihydroergocriptine.

As with the expression of CYP isoforms and the expression of genes for the PXR and RXR nuclear receptors, it is very likely that the overall impact of fescue toxicosis on hepatic antioxidant enzymes is at least, in part, a result of decreased nutrient and caloric intake, rather than a direct effect of endophytic toxins. Cho et al. (1981) reported reduced glutathione concentrations in starved rats, and Marczuk-Krynicka et al. (2003) observed reduced expression of catalase and CuZn-SOD but not GPx in rats deprived of feed. The effects of reduced feed intake on PH-GPx have not been elucidated. This effect was incorporated into this preliminary study in an effort to determine both the direct and indirect effects of fescue toxicosis on this particular system.

Effects on hepatocellular proliferation

To evaluate the adverse hepatic effects of short-term exposure to ergot alkaloids associated with fescue toxicosis, in the absence of histological abnormalities, PCNA was measured as an indicator of hepatocellular proliferation. It was evident that fewer hepatocytes were replicating in the livers of rats exposed to ergot alkaloids. It is very

likely that oxidative stress, in part due to the direct effects of endophytic toxins and also as a physiological response to decreased nutrient and caloric intake, contributed to the diminished hepatocellular proliferation observed in these rats.

6. CONCLUSION

The results of the present study show the effects of ergot alkaloids on the hepatic expression of CYP and antioxidant proteins in rats and offer explanation as to how those alterations might contribute to the clinical signs of fescue toxicosis. CYP protein expression was up-regulated in as a detoxification mechanism and, as a response to decreased nutrient and caloric intake. In contrast, there was diminished expression and activity of hepatic antioxidant enzymes. With the supposition that up-regulation of hepatic CYP isoforms will result in increased reactive xenobiotic metabolites, including ROS, it is likely that the reduced antioxidant activity predisposes ergot alkaloid-exposed animals to hepatic oxidative stress. The results of the present study emphasize the potential role of even short-term endophytic toxin-associated oxidative damage in the pathogenesis of fescue toxicosis.

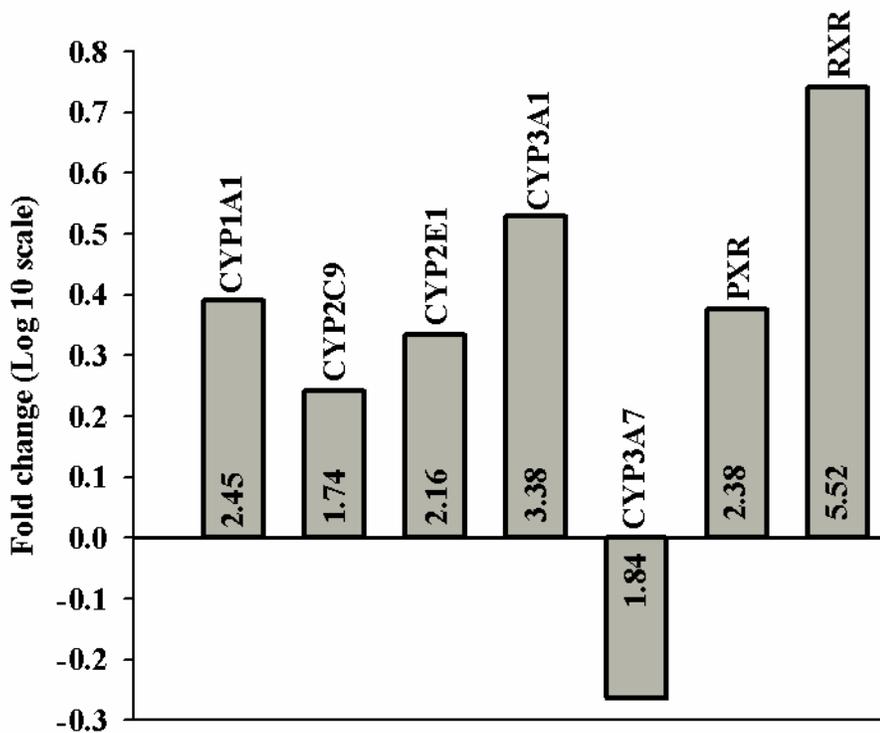


Figure 5. 1. The effects of dietary exposure to fescue toxicosis-associated ergot alkaloids on hepatic CYP and nuclear receptor gene expression are shown. Columns above baseline represent up-regulated CYP genes, whereas those below baseline represent down-regulated CYP genes in livers of rats exposed to E+ versus E- diet. Values within columns represent relative fold changes in gene expression of CYP isoforms in the livers of rats fed E+ versus E- diet. Gene expression of all CYP isoforms and nuclear receptors is significantly different ($p \leq 0.05$) in E+ compared to E- rats.

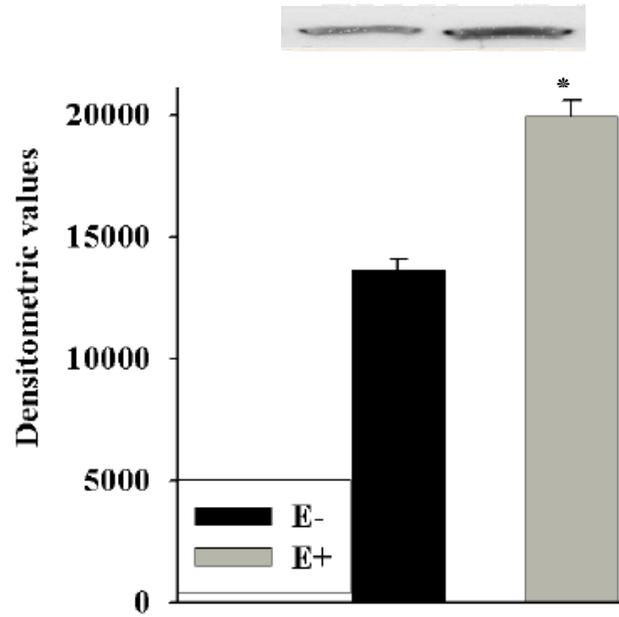


Figure 5. 2. The induction of expression of CYP3A1 protein in the livers of rats exposed to diet containing the ergot alkaloids associated with fescue toxicosis is shown. Protein expression of CYP3A1 is significantly increased ($p < 0.01$) in E+ fed compared to E- fed rats.

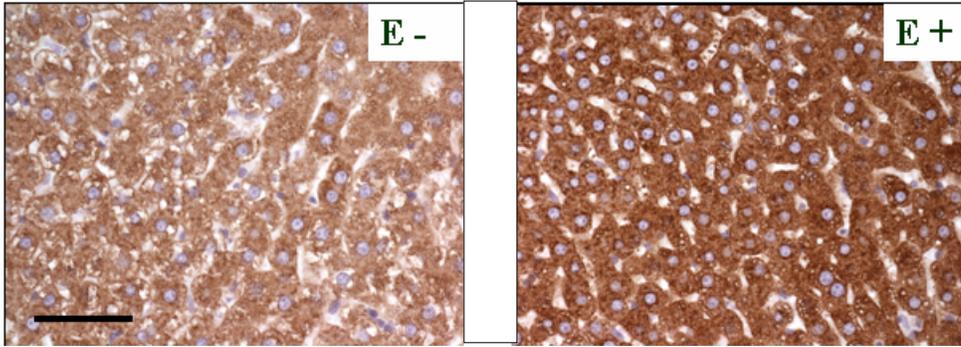


Figure 5. 3. The effect of dietary exposure to ergot alkaloids associated with fescue toxicosis on immunohistochemical staining for CYP3A1 in hepatic sections is shown. Sections of livers from rats exposed to fescue toxicosis-associated ergot alkaloids consistently stained more intensely for CYP3A1 (40X magnification; the calibration bar represents 60 μ m).

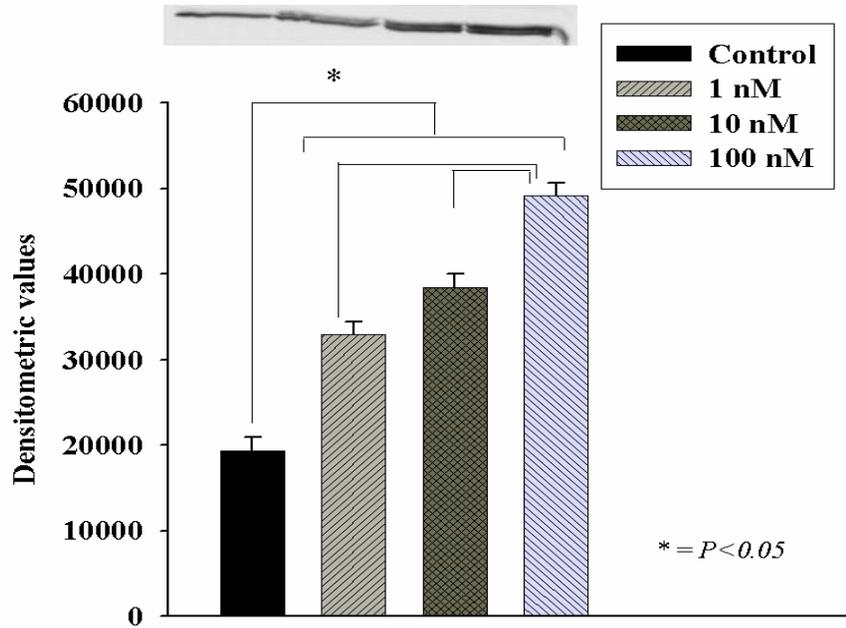


Figure 5. 4. The dose-dependent induction of CYP3A1 by ergovaline (i.e., the direct effects of ergovaline on CYP3A1 protein expression) in primary hepatocellular cultures is shown. A significant induction ($p < 0.01$) in CYP3A1 protein expression was observed in all ergovaline-treated primary hepatocellular cultures compared to untreated cells. All comparisons except between 1 nM and 10 nM are significantly ($p < 0.05$) different.

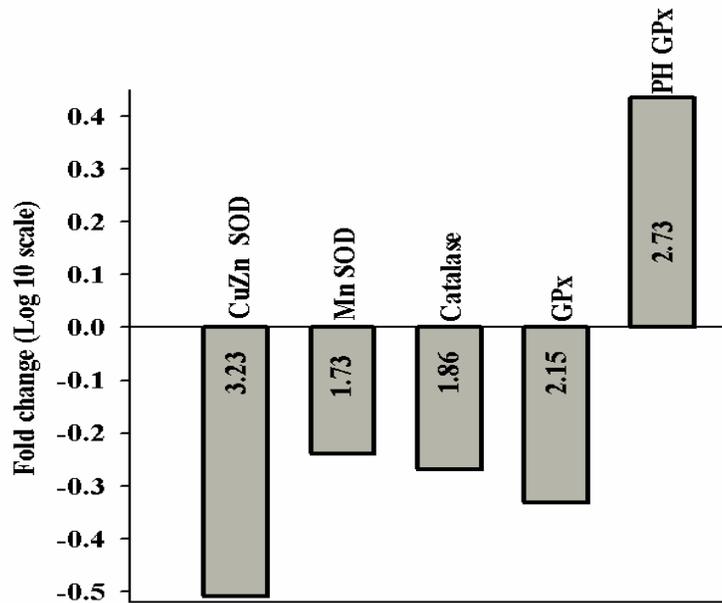


Figure 5. 5. The statistically significant effects of dietary exposure to ergot alkaloids associated with fescue toxicosis on the hepatic gene expression of antioxidant enzymes are shown. Columns above baseline represent up-regulated genes corresponding to antioxidant enzymes, whereas columns below baseline represent down-regulated genes for antioxidant enzymes in the livers of rats fed E+ versus E- diet. Values within columns represent relative fold changes in hepatic gene expression in rats fed E+ versus E- diet. Gene expression of all antioxidants is significantly different ($p \leq 0.05$) in E+ compared to E- rats.

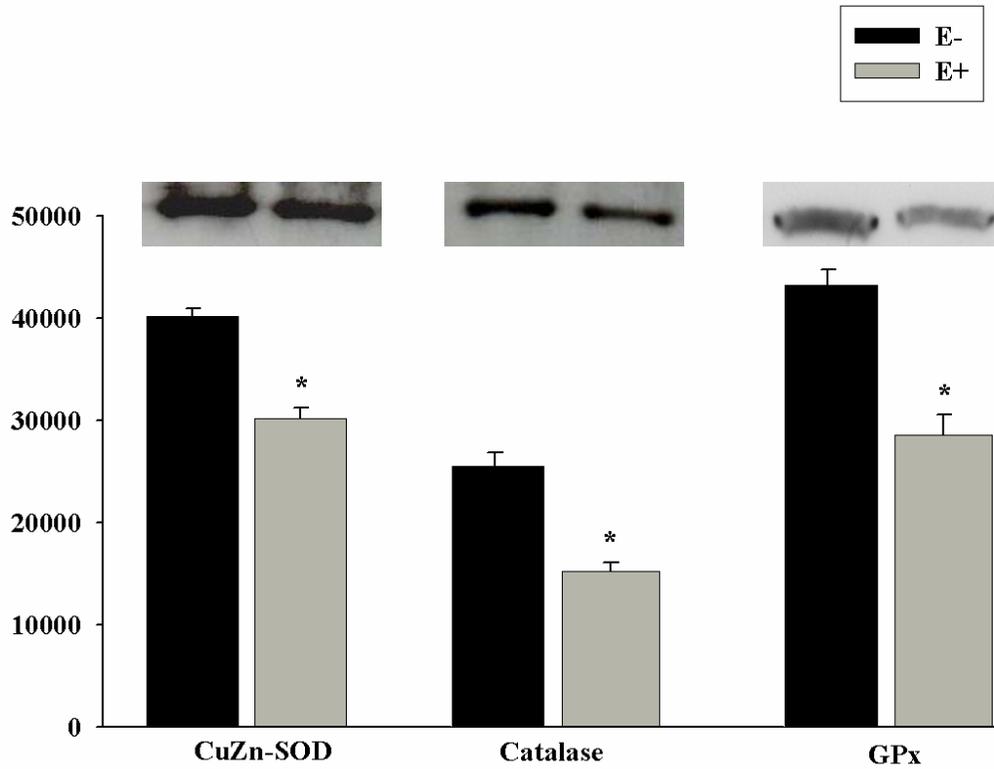


Figure 5. 6. The decreased expression of various antioxidant enzymatic proteins in the livers of rats exposed to diet containing the ergot alkaloids associated with fescue toxicosis is shown. Protein expression of all antioxidants is significantly reduced ($p \leq 0.05$) in E+ compared to E- rats.

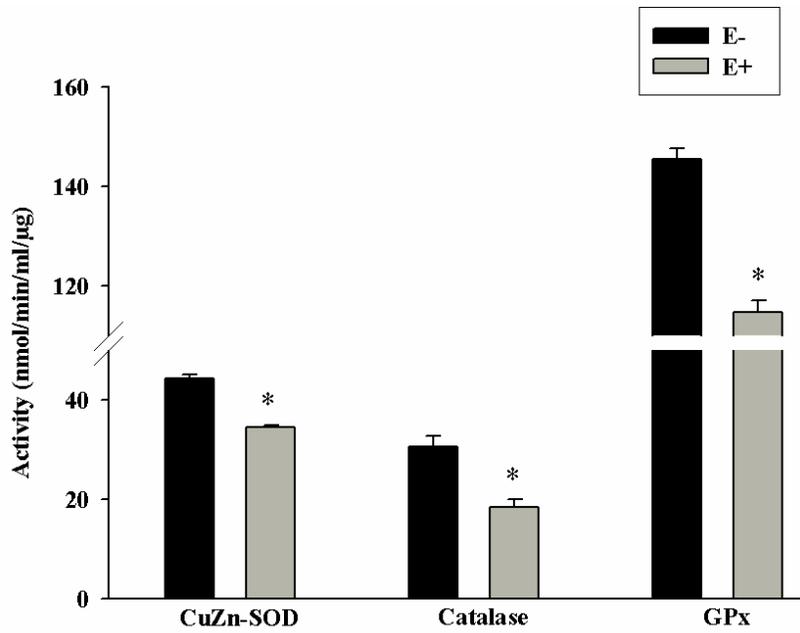


Figure 5. 7. The decreased antioxidant activity in the livers of rats exposed to diet containing the ergot alkaloids associated with fescue toxicosis is shown. Activities of all antioxidants are significantly reduced ($p \leq 0.05$) in E+ compared to E- rats.

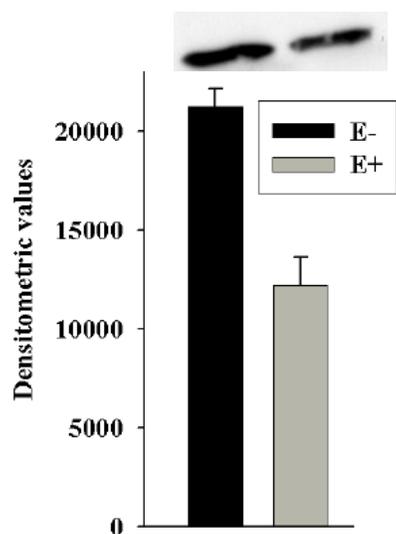


Figure 5. 8. Decreased protein ($p = 0.02$) expression of PCNA (i.e., the decreased hepatocellular proliferation) in the livers of rats exposed to diet containing the ergot alkaloids associated with fescue toxicosis is shown.

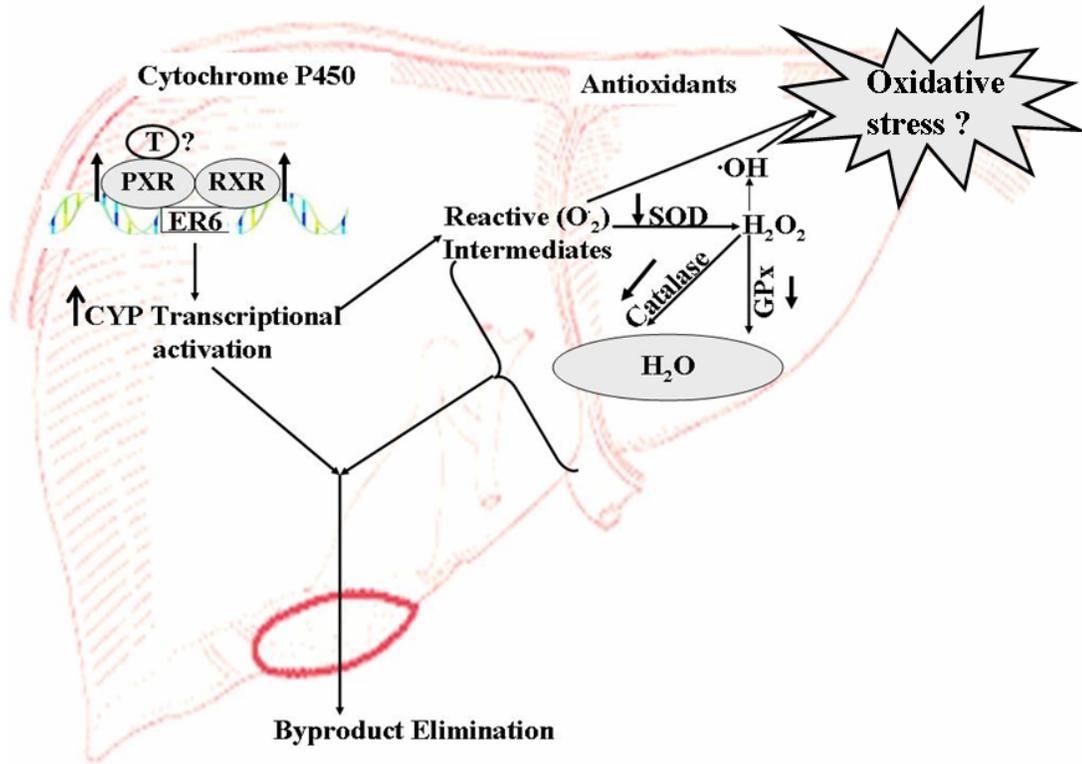


Figure 5. 9. The potential roles of ergot alkaloid-induced CYP overexpression, as well as decreased responsiveness to oxidative stress, in the pathogenesis of hepatic insult associated with fescue toxicosis are summarized. While it is apparent from the experimental results that some aspects of the observed ergot alkaloid-induced effects (i.e. up-regulated CYP expression) are directly associated with endophytic toxin exposure, other genomic and proteomic alterations (i.e., nuclear receptor and antioxidant enzyme expression) are likely caused by decreased nutrient and caloric intake and, therefore, indirectly associated with exposure to ergot alkaloids.

Table 5. 1. Details of CYP and nuclear receptor primers used for RT-PCR

Gene	Genbank No.	Forward primer	Reverse primer
CYP1A1	P00185	GGCCGACTGGAGGTCTTTC	GGTGACACATTAATCCATTGTC
CYP1A2	A61400	AAGCGCCGGTGCATTG	GCAGGAGGATGGCTAAGAAGAG
CYP2C6	P05178	GAGCCGCATGCCCTACAC	CAGGTTGGTAGGAATGAGGTCAA
CYP2C9	P08683	CGGCGTTTCTCCATCATGA	TCTTGAATACGGTCCTCAATGGT
CYP2E1	NP113731	TGACTTTGGCCGACCTGTTC	TGAGGATCAGGAGCCCATATCT
CYP3A1	JX0334	GGAGGTGCTCTTGCCATCTG	CACCCCCACATGTAGTGCATAA
CYP3A2	A25222	AAGCGCCGGTGCATTG	TGCAGGAGGATGGCTAAGAAG
PXR	NC007859	GCTACCTGCGGTGTTTCACA	CCAGATTGCTGCCGTCTTCTC
RXR	NC005119	CTTCTCCCACCGCTCCATAG	CATCTGCATGTCACGCATCTT

Table 5. 2. Details of antioxidant enzyme primers used for RT-PCR

Gene	Genbank No.	Forward primer	Reverse primer
Catalase	NM012520	CCCAGAAGCCTAAGAATGCAA	GCTTTTCCCTTGGCAGCTATG
GST	I38615	ATGGAGAAATAGGCAGTTCATGTG	CCGGCCCAGATCTCCTCTA
GPx	P04041	CGATACGCCGAGTGTGGTTT	GCTCCTGCCTCCCGAACT
Heme oxygenase - 1	NP034572	CGCATGAACACTCTGGAGATGA	AGGCGGTCTTAGCCTCTTCTG
Nitric oxide synthase	S16233	CGGTTCACAGTCTTGGTGAAAG	ACGCGGGAAGCCATGAC
PHGPx	P36970	TGAGGCAAAACCGACGTAAAC	CGTAAACCACACTCGGCGTAT
CuZn-SOD	JC1192	CTCAGGAGAGCATTCCATCATTG	TTTCCACCTTTGCCCAAGTC
MnSOD	P07895	AGCCTCCCTGACCTGCCTTA	GCATGATCTGCGGTTAATG
GAPDH	NM023964	TGCCAAGTATGATGACATCAAGAAG	AGCCAGGATGCCCTTTAGT

CHAPTER VI

EFFECTS OF ACUTE HEAT STRESS ON ERGOT ALKALOID-INDUCED ALTERATIONS IN HEPATIC GENE EXPRESSION

ABSTRACT

Intake of ergot alkaloids associated with endophyte-infected fescue (E+) has adverse effects on animal health and productivity, which is collectively referred to as fescue toxicosis. These effects are exacerbated under hot and humid environmental conditions. A rat model for was used to evaluate the effect of endophytic toxins on hepatic gene expression under acute heat stress (HS). Rats implanted with telemetric transmitters (n = 12) were fed *ad libitum* an E+ diet and maintained under thermoneutral (TN) conditions (21°C) for five days, followed by TN or HS (31°C) conditions for three days. Core temperature (T_c) was monitored continuously and feed intake (FI) and body weight (BW) were measured daily. Both E+ and HS induced alterations in hepatic genes were evaluated using DNA microarrays in flash-frozen samples of liver collected at the end of the exposure. Protein expression of cytochrome P450 3A1 (CYP3A1), the enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the rate of hepatocytic apoptosis using the TUNEL assay were also determined in these samples. As expected, intake of E+ reduced FI and BW from

pretreatment levels under both TN and HS conditions, with a greater reduction occurring during acute HS. Core temperature at TN did not change from pretreatment levels but increased during HS, and, as anticipated, serum prolactin decreased with E+ treatment but increased during HS. Genes associated with apoptosis were upregulated following acute HS, and genes involved with ATP synthesis, antioxidant function, chaperone activity, xenobiotic metabolism and immune function, were down-regulated with acute HS. The TUNEL positive hepatic nuclei and CYP3A1 protein expression increased in E+HS with a reduction in SOD and GPx activities. The results of the present study indicate that the anticipated exacerbation of the adverse physiological effects of ergot alkaloid exposure by acute heat stress are associated with upregulation of genes involved in apoptosis and suppression of the expression of genes associated with ATP synthesis, immune system function and chaperone, xenobiotic metabolizing and antioxidant activities, which could ultimately increase the stress resulting in various pathological abnormalities. The added effects of acute heat stress on ergot alkaloid-induced alterations in hepatic gene expression very likely play an important role in the pathogenesis of the adverse effects of ergot alkaloids on various animal species.

2. INTRODUCTION

Consumption of ergot alkaloids by cattle, produced by the fungus *Neotyphodium coenophialum*, results in a disease syndrome called fescue toxicosis. One of the characteristic features of fescue toxicosis is peripheral vasoconstriction resulting from the interactions of ergot alkaloids with alpha-2 adrenergic, serotonergic and dopaminergic

receptors (Oliver, 2005). Other characteristics of fescue toxicosis include reduced feed intake, and both serum prolactin and cholesterol levels (Porter and Thompson, 1992; Aldrich et al., 1993; Paterson et al., 1995). Fescue toxicosis-related problems get exacerbated during heat stress as the animal's ability to dissipate excess body heat is impaired, resulting in hyperthermia and reduced growth rate (Spiers et al., 2005a). The clinical signs of fescue toxicosis in cattle appear to be primarily dependent on the level of heat (Hemken et al., 1981). Rats also exhibit the characteristic clinical signs of fescue toxicosis and are sensitive to the adverse effects of the ergot alkaloids associated with fescue toxicosis under both thermoneutral (TN) and heat stress (HS) conditions (Spiers et al., 2005a).

The liver is one of the most important organs for the regulation of carbohydrate, lipid, protein and xenobiotic metabolism, and has been identified as a target organ of fescue toxicosis (Oliver, 1997). Long-term intake of E+ diet in cattle, increases liver-specific enzymes (Piper et al., 1991), and a component of the fescue toxicosis response in rats is reduced liver weight (Settivari et al., 2006). Chestnut et al. (1992) noted that this reduction in liver weight in rats fed an E+ diet is also observed in rats fed a control diet at the same caloric level (i.e., pair-fed group). Although it is possible that the clinical signs associated with fescue toxicosis are due to both direct and indirect (i.e., reduced caloric intake) effects on hepatic function, the objective of this preliminary study was to determine the overall impact of fescue toxicosis on hepatic gene expression under short-term heat stress conditions. A previous study at TN revealed that various genes associated with energy metabolism, and both antioxidant and immune systems were down-regulated, whereas genes involving xenobiotic metabolism and gluconeogenesis were up-regulated

in rats fed an E+ diet for five days (Settivari et al., 2006). This research is one of the first attempts to determine changes in hepatic gene expression associated with fescue toxicosis during short-term heat stress. It was hypothesized that the exacerbated clinical signs of ergot alkaloid exposure observed in rats following short-term heat stress would be associated with significant genomic alterations consistent with the pathogenesis of fescue toxicosis in animal species.

3. MATERIALS AND METHODS

Animals and treatment diets

Male Sprague-Dawley rats (n = 12; 42 days of age at start; Charles River Laboratories, Willington, MA), were maintained on a 12 h light/dark cycle and at TN as described earlier (Spiers et al., 2005a; Settivari et al., 2006). Feed and water were provided *ad libitum* throughout the study. Uninfected control (E-, containing endophyte free fescue seed) and treatment diet compositions were as previously described, with the treatment diet adjusted to provide a specific amount of ergovaline (EV) (Spiers et al., 2005a), which is considered to be the major ergopeptine alkaloid in E+ seed. Ergovaline content in E+ seed was 4,100 ppb on a DM basis, as measured by HPLC (detection limit = 50 ppb and CV = 7 %; Rottinghaus et al., 1993). All diets were stored at 4°C prior to use. Animal procedures followed guidelines for humane treatment set by the University of Missouri Animal Care and Use Committee.

Treatment period and sample collection

The experimental schedule consisted initially of an 8 day recovery period after i.p. implantation of telemetric temperature transmitters (Model VM-FH; Mini-Mitter Company, Inc., Bend, OR) to monitor Tc. This was followed by a 5 day pretreatment period, during which Tc, FI and BW were collected to establish a baseline for statistical analysis (Spiers et al., 2005a). During recovery and pretreatment periods, rats were fed the E- diet, whereas during the 8 day treatment period, the rats were fed the E+ (91.5 μg of EV.kg of BW⁻¹.day⁻¹) diet. At the end of 5 days of treatment, rats were divided into two groups, one maintained under continuous HS (31°C) and the other continuing at TN for three additional days. During treatment Tc was collected at 10 minute intervals, with daily measurement of FI and BW at 0800 hour. At the end of the study, rats were anesthetized by i.m. injection of a xylazine (13 mg/mL)-ketamine (87 mg/mL) cocktail (0.1 mL/100g) and exsanguinated, and liver was collected and weighed. A transverse section from the middle lobe of liver (~5 g) was fixed in 10% neutral buffered formalin for CYP3A1 immunohistochemistry (IHC) and terminal dUTP nick-end labeling (TUNEL), and another portion (~5 g) was snap-frozen in liquid nitrogen for microarray, real time-PCR, and antioxidant enzyme activity analysis. Serum prolactin (PRL) concentrations were determined using rat prolactin enzyme immunoassay kit (SPI – BIO, Massy Cedex, France) as previously reported (Duhau et al., 1991).

Statistical analysis of physiological responses

Daily average values for Tc, FI and BW were computed as a function of time and treatment group. These parameters were analyzed by repeated measures ANOVA (Mixed procedure) of SAS (SAS Inst., Inc., Cary, NC) standard least square model fit (JMP[®],

SAS Institute Inc., Cary, NC) using treatment, time and animal as class variables to determine overall treatment and time-related differences, and treatment X time interactions. Enzyme activities and densitometric values for various parameters were analyzed for significance using one-way analysis of variance and proc univariate analysis of SAS. If a significant difference existed for any comparison, then a Tukey - Kramer multiple comparisons test (Steele and Torrie, 1980) was performed ($\alpha = 0.05$) to determine specific effects. All data is reported as mean \pm standard error of mean. A p-value less than 0.05 were considered significant between E- and E+ rats.

Microarray slide preparation

Microarray slides were prepared by printing 27,000 oligonucleotides (70 mer probes with an amino modification on the 5' end) specific to mouse liver (Oligator MEEBO mouse genome set, Illumina Inc., Hayward, CA) on GAPS II glass slides (Corning Incorporated, Corning, NY) using a 32-pin robotic microarray printer. Prior to hybridization, the microarray slides were re-hydrated to increase the spot size and spread the DNA more uniformly within each spot. Following the re-hydration for 30 seconds at 55°C, the slides were snap-dried on a 100°C hot plate surface for 5 seconds. Then the slides were UV cross-linked using UV cross-linker (600 mJoules). Microarray slides were then incubated in 0.2% I-block (Tropix/Serva, Heidelberg, Germany) in 1 \times PBS buffer for at least one hour at 42°C to decrease background and increase specificity. The slides were then washed with 0.2% SDS once and water twice (three minutes per wash). The slides were then dried by centrifugation and stored in dark until used.

RNA extraction

Total RNA was extracted from the liver samples using an RNeasy Midi Kit (Qiagen Inc., Valencia, CA), purified using DNase-1 (Ambion Inc., Austin, TX) and phenol:chloroform:isoamyl alcohol (25:24:1), and concentrated using Microcon YM30 filters (Millipore Corp., Bedford, MA) as described earlier (Settivari et al., 2006). The quality and integrity of the purified RNA was checked through agarose gel electrophoresis and the quantity was measured using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE; Flanagan, 2005). The purified RNA samples were preserved at -80°C until used.

Microarray hybridization

A loop design was used for microarray experiments, with six biological replicates (six animals/treatment group) and three technical replicates (each RNA sample repeated on three different arrays). Concentrated and purified total RNA (15 µg) from each rat liver was reverse-transcribed to cDNA using oligo dT (IDT DNA, Coralville, IA), random hexamers (IDT DNA), 50 x amino-allyl dUTP (Sigma Chemicals, St. Louis, MO), and reverse transcription (StrataScript RT, Stratagene, La Jolla, CA). Resulting cDNA was purified using Microcon-30 filters (Millipore) and conjugated with either Cy3 or Cy5 mono reaction dyes separately by incubating in the dark for one hour. Conjugated cDNA was separated from free, unconjugated dye using a Qia-quick PCR purification kit (Qiagen) following the manufacturer's instructions. The two cDNA samples, labeled with Cy3 and Cy5 dyes, were mixed together and dried. The dried cDNA pellet was resuspended with the hybridization mixture, which consisted of 1 × 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), poly A (10 µg/µL; Stratagene), rat Cot-I DNA

(3.2 $\mu\text{g}/\mu\text{L}$), 20 \times standard saline citrate (SSC; Stratagene), and 10% sodium dodecyl sulfate (SDS). The slides were incubated in a water bath (60°C) for 12 to 16 h. After incubation, the slides were rinsed in wash solution I (20 \times SSC, and 10% SDS) and then in wash solution II (20 \times SSC).

Microarray slide scanning and analysis

Microarray slides were scanned using a GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA) at five μm resolution, and the image was analyzed using GenePix Pro 4.0.1.12 software (Axon Instruments Inc., Union City, CA). The resulting files and the images were linked together and stored in the local BioArray Software Environment (BASE) database (Saal et al., 2002). Microarray raw data files were deposited with the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/>). The serial entry number for the microarray data was GSE8390, and the platform number was GPL5357. Each hybridization in the microarray experiment was given the following serial numbers: GSM202415, GSM206879, GSM206881, GSM206885, GSM206897, GSM206901, GSM206903, GSM206905, GSM206906, GSM206983, GSM206984, GSM207053, GSM207096, GSM207097, GSM207098, GSM207099, GSM207100, GSM207101, GSM207102, GSM207103, GSM207105, GSM207106, GSM207107, GSM207115, GSM207117, GSM207118, GSM207119, GSM207126, GSM207130, GSM207135, GSM207141, GSM207143.

Self-self hybridization

Five self-self hybridizations were performed to measure the variation in gene expression due to technical errors. In these, the same rat was used for different RNA

isolations, labeling, and hybridizations. Slides were hybridized, scanned, and analyzed as just described. Means and standard deviations for Cy5 and Cy3 ratios were calculated for the five hybridizations, and a significant threshold was set as mean \pm 2 SD (i.e., \pm 1.4). Genes with expression above or below this threshold were considered as up or down-regulated, respectively, and were used for further analysis.

Statistical analysis of microarray data

Microarray data was first filtered to remove all the blank spots. The background corrected median intensities were normalized using pin-based LOWESS normalization within BASE. The microarray spot intensities were first log₂ transformed and genes with greater than 20% missing values were filtered using CLUSTER 3.0 software. Missing values for each gene was imputed using k-nearest neighbor method (k = 20) using significant analysis of microarrays (SAM) software. The normalized intensities were then inputted into the software package MAANOVA (Wu et al., 2003) to model the data and run statistical analyses. A two-stage ANOVA model (Wolfinger et al., 2001) was applied to the microarray data using JMAANOVA (Wu et al., 2002) software. The first stage was the normalization model to remove the effect of array and dye at the across gene level

$$y_{gnljk} = \mu + A_i + D_k + (AD)_{ik} + \varepsilon_{gnljk}$$

The second stage was the gene-specific model in which the effects of animal-gene interaction and array-gene interaction were modeled as random effects.

$$\varepsilon_{gnljk} = (GV)_{gt} + (GD)_{gtk} + r_{gnljk}$$

For these models $y_{gnljk} = \log_2(w_{gnljk})$, w is the observed median intensity for each color channel by gene, μ is the model grand mean, A is the array, D is the dye, G is the gene, V is the animal, and ε and r are the residuals from the linear models.

A permutation F_s test (an F test designed specifically for microarray data) (Cui et al., 2005) was run to test the significance of sample effect for each probe. Probes with a permutation F_s test p value less than 0.05 were regarded as significantly differentially expressed across samples.

The first stage was normalization model to remove the effects of array and dye. The residual from this model was used in the second stage analysis, which is a gene specific model, where the effects of sample-gene interaction and array-gene interaction were modeled with both effects at random. To test the significance of sample effect for each probe (500 permutations), the F_s test (Cui et al., 2005) was used. Genes, with an adjusted permutation F_s test (False Discovery Rate; FDR) values less than 0.05, were regarded as significantly differentially expressed across samples. This differentially expressed gene list was then filtered to remove genes with a fold change of less than 1.4 (value obtained from mean \pm 2 SD of five self-self hybridizations).

A second type of microarray analysis was performed which measures the gene expression across each individual animal (gene by animal model) using JMAANOVA program. Values obtained from this analysis were used to cluster similarly expressed genes using TIGR multi-experiment viewer software (TM4-Mev; Saeed et al., 2003). The hierarchical cluster tree obtained was confirmed using bootstrap (Kerr and Churchill, 2002) analysis. The DAVID (Dennis et al., 2003) and Entrez-Gene (<http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>) from the National Center for Biotechnology Information were used to find the gene annotation and the functions of differentially expressed genes.

Quantitative real-time PCR (qRT-PCR)

Two-step qRT-PCR was used to confirm expression patterns of differentially expressed genes in the study. Expression profile of eight randomly selected genes (Table 6. 1) was measured with qRT-PCR. From each rat, 10 µg of total RNA was reverse transcribed using Stratascript RT (Stratagene, La Jolla, CA) with oligo dT and random hexamer primers. Then 6.25 ng of cDNA were added to a 25 µL PCR reaction to get a final concentration of 0.25 ng/µL of cDNA in a SYBR green assay (Applied Biosystems, Warrington, UK). Forward and reverse primer final concentrations were 100 nM in the SYBR green assay. Primers were designed using Primer3 program with an annealing temperature of 60°C and amplification size of less than 250 base pairs. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the endogenous control gene in the qRT-PCR experiments. Thermal cycling was carried out with an ABI Prism 7500 sequence detection system (Applied Biosystems Warrington, UK) under factory default conditions (50°C, 2 min; 95°C 10 min; and 40 cycles at 95°C, 15 sec; 60°C 1 min). Each gene was measured in triplicates and the formation of single PCR product was confirmed using dissociation curves. Negative controls, which consisted of all the components of PCR mix except cDNA, were used for all the primers. The relative quantification of gene expression changes were recorded after normalizing for GAPDH gene expression computed by using the $2^{-\Delta\Delta CT}$ method (user manual #2, ABI Prism 7700 SDS). In the $2^{-\Delta\Delta CT}$ analysis, the threshold cycle (C_T) from E+TN rats was used as a calibrator sample. Statistical analyses of the data were performed by comparing E+HS C_T with E+TN C_T for each gene using a two-tailed t-test with unequal group variance.

Hepatic CYP3A1 protein expression

Protein expressions were determined using a standard immunohistochemistry protocol as described earlier (Bowles et al., 2004). Briefly, liver samples were fixed in neutral buffered 10% formalin for a minimum of 24 hours, embedded in paraffin, and sectioned serially at 4 μm thicknesses. Sections were floated onto positively charged slides (Thomas Sci, Swedesboro, NJ), deparaffinized, and steamed in citrate buffer at pH 6.0 for 30 min to achieve antigen retrieval. Sections were then incubated with avidin-biotin two step blocking solution to inhibit background staining and in 3% H_2O_2 to inhibit endogenous peroxidase. The sections were incubated overnight at 4°C with a 1:500 dilution of primary rabbit monoclonal antibody to CYP3A1 (Santa Cruz, CA). Following incubation, sections were incubated with biotinylated anti-rabbit secondary antibody in PBS containing 15 mM sodium azide and peroxidase-labeled streptavidin (Dako LSAB+ kit, peroxidase, K0690). Images were captured at 400 x magnification (Insight Color Camera Sterling Heights, MI).

Hepatic anti-oxidant enzyme activities

Rat liver samples of both groups were homogenized as described earlier and total protein concentrations were determined using a Bradford protein assay kit (BioRad). The enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were assayed spectrophotometrically following manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). The specific activity of hepatic GPx, SOD, and CAT was expressed as unit μg^{-1} liver total protein. Glutathione peroxidase activity was measured indirectly by a coupled reaction with glutathione

reductase. Glutathione peroxidase reduces hydroperoxides and result in production of oxidized glutathione, which in turn is recycled to its reduced state by glutathione reductase and NADPH. Glutathione reductase oxidizes NADPH to NADP⁺, and is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A₃₄₀ is directly proportional to the GPx activity in the sample. One unit was defined as the amount of enzyme that oxidized 1.0 nmol of NADPH to NADP⁺ per minute at 25°C. Hepatic SOD activity was assayed using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The enzyme activity (one unit) was measured as the amount of enzyme required to exhibit 50% dismutation of superoxide radicals. Hepatic catalase has peroxidatic activity, by which it acts on low molecular weight alcohols to convert them into formaldehyde. The formaldehyde formed was measured spectrophotometrically. Purpald, which gives maximal absorbance at 540 nm was used as a chromogen. One unit was defined as the amount of enzyme that produced 1.0 nmol of formaldehyde per minute at 25°C.

Apoptosis assay

Paraffin-fixed liver sections from both treatment groups were used to identify the apoptosis rates in E+HS vs E+TN rats using TUNEL assay as previously described (Hao et al., 2003). Fixed liver sections were deparaffinized using xylene, 100, 90 and 70% ethanol, washed in PBS with triton X and incubated in TUNEL reaction medium (in situ Cell Death Detection Kit, AP; Roche diagnostics corporation, Indianapolis, IN) at 37°C for one hour in the dark. Then the sections were washed in PBS, transferred onto 2 µg/ml of DAPI (4', 6-diamidine-2'-penylindole dihydrochloride; Roche), and mounted on the slides with ProLong Antifade kit (Molecular Probes, Eugene, OR). As negative controls

for TUNEL, fixed hepatic sections were incubated in TUNEL reaction medium which lacked the TdT enzyme solution. Liver sections were studied with an epifluorescent microscope (Nikon, Tokyo, Japan) using detection for TUNEL and DAPI to visualize apoptotic cells and DNA, respectively. The number of apoptotic nuclei and total number of nuclei were determined for 500 cells randomly.

4. RESULTS

Physiological responses to fescue toxicosis

Pretreatment values at TN (Days -2 to 0)

Pretreatment group averages for Tc were not different ($p = 0.43$). Likewise, average group feed intake ($p = 0.76$) and water intake ($p = 0.90$) did not differ during pretreatment period. Feed conversion efficiency (FCE) was calculated as the ratio of changes in body weight to FI multiplied by 100. There were no differences ($p = 0.10$) between group pretreatment FCE. Finally, there were no differences between group values for body weight ($p = 0.19$) and growth rate ($p = 0.07$) as a function of treatment during pretreatment period.

Physiological responses to treatment diet at thermoneutrality (Days 1 to 5)

During TN treatment period, intake of the E+ diet did not produce significant change ($p > 0.43$) in daily average Tc of E+TN and E+HS from pretreatment level. Similarly, Tc between E+TN and E+HS was not different ($p = 0.20$) during this period. Furthermore, time-related (day) changes in Tc of E+TN and E+HS rats were absent ($p > 0.05$) during TN treatment period.

Intake of an E+ diet produced a reliable decrease ($p < 0.01$) in feed intake of E+TN (59.48 g/kg BW) and E+HS (47.47 g/kg BW) rats from Day -1 of pretreatment, within the first 24 hours. By the end of TN treatment period (Day 5), E+TN (21.41 g/kg BW; $p < 0.01$) and E+HS (13.88 g/kg BW; $p = 0.03$) rats exhibited signs of adaptation with an increase in FI from Day 1 level (Figure 6. 2a). As expected, no difference ($p = 0.58$) in FI was observed between E+TN and E+HS rats during this period.

The E+ diet intake, resulted in a reduction in FCE of E+TN (47.65 g BW/g FI: $p < 0.01$) and E+HS (23.50 g BW/g FI: $p = 0.04$) rats within the first 24 hours from Day -1 level. However, by Day 2, complete adaptation occurred in both treatment groups and the values returned to pretreatment level. The FCE values were similar ($p = 0.51$) between the treatment groups and time-related changes were absent ($p = 0.17$) during this period (Figure 6. 2b).

Water intake values were similar ($p > 0.79$) to pretreatment level in both groups during this period. Likewise, the values were similar ($p = 0.30$) between the treatment groups with no time-related changes ($p = 0.21$) occurring in either group (Figure 6. 1b).

Intake of an E+ diet did not change body weight of E+TN and E+HS rats from pretreatment level (Figure 6. 3a). However, the growth rate for both groups was decreased ($p < 0.01$) within the first 24 hours from Day -1 level (Figure 6. 3b). Growth rate remained low ($p < 0.05$) in both groups even at the end of Day 5. Body weight and growth rate did not change ($p = 0.47$) between E+TN and E+HS rats during this period. Time-related changes were absent for body weight, but growth rate in both groups decreased ($p = 0.05$).

Physiological responses to treatment diet during short-term heat stress (Days 6 to 8)

Core temperature of E+TN rats did not differ ($p = 0.14$) from Day 5 to Day 6, and remained at the same level until the end of study (Day 8; $p = 0.13$). In contrast to E+TN rats, there was a reliable increase (0.46°C ; $p < 0.01$) in T_c of E+HS rats from Days 5 to 6 (Figure 6. 1a). There was a continuous increase in T_c of E+HS rats until end of Day 8, with an additional increase ($p < 0.01$) of 0.21°C above Day 6 level. There was a time-related change in T_c during HS, with the values increasing ($p < 0.01$) for E+HS rats from Day 6 to 8. During HS period, T_c was greater ($p < 0.01$) in E+HS compared to E+TN rats.

The E+TN rats continued to exhibit signs of adaptation in FI on Day 6, with an additional increase ($p = 0.08$) of 7.09 g/kg BW above Day 5 level. The values remained at this level until the end of study (Figure 6. 2a). In contrast, feed intake of E+HS rats decreased (13.06 g/kg BW; $p < 0.01$) on Day 6 from Day 5 level. Partial signs of adaptation (7.70 g/kg BW; $p = 0.06$) occurred in E+HS rats on Day 8. The E+HS rats exhibited a significant reduction (22.69 g/kg BW; $p < 0.01$) in FI during this period compared to E+TN rats.

Feed conversion efficiency was similar ($p = 0.72$) in E+TN rats on Days 5 and 6, and remained at this level until the end of study (Day 8; $p = 0.38$). In E+HS rats, however, FCE values decreased (80.66 g BW/g FI; $p < 0.01$) below Day 5 level within the first 24 hours of heat exposure. The E+HS rats exhibited signs of adaptation in FCE by Day 7 (59.28 g BW/g FI; $p < 0.01$) and by Day 8 the values reached TN level. During

this period, FCE values decreased (59.98 g BW/g FI; $p < 0.01$) in E+HS versus E+TN rats. Similarly, time related changes were observed for FCE during this period, with the values decreasing in E+HS group from TN level; whereas no such changes occurred in E+TN rats.

Water intake of E+TN rats did not change ($p = 0.96$) from Day 5 to Day 6, and the values remained at the same level ($p = 0.40$) until the end of Day 8. In contrast to E+TN rats, there was a reliable increase (182.15 ml/kg BW; $p < 0.01$) in WI of E+HS rats by Day 6. The WI values in E+HS rats remained elevated until the end of study with no signs of adaptation. During this period, WI values were significantly increased (182.91 ml/kg BW; $p < 0.01$) in E+HS compared to E+TN rats. Time-related changes were observed for FCE during this period, with the values increasing in E+HS group from TN level, with no such changes occurring in E+TN rats.

Body weight gain and growth rate did not change ($p > 0.60$) in E+TN rats on Day 6 from Day 5 level. By Day 8, however, complete adaptation occurred in the growth rate of E+TN rats and the values returned to pretreatment level. In contrast, body weight of E+HS rats exhibited only a tendency towards reduction ($p = 0.12$) on Day 6 from Day 5 level. The growth rate of E+HS rats decreased ($p < 0.01$) on Day 6 from Day 5 level. The E+HS rats exhibited signs of adaptation ($p < 0.01$) in growth rate by Day 7 from Day 6 and the values returned to preheat level by Day 8; however signs of adaptation were absent in the body weight of E+HS rats during this period. Body weight and growth rate of E+HS rats was reduced ($p < 0.03$) compared to E+TN rats during this period.

Average wet weights of liver in g/kg BW decreased ($p = 0.03$) in E+HS (31.2 ± 1.4) compared to E+TN (35.2 ± 1.4) rats. In contrast, serum prolactin ng/ml increased ($p = 0.04$) in E+HS (19.0 ± 1.6) compared with E+TN (11.4 ± 1.6) rats.

Genomic responses to fescue toxicosis

Microarray analysis identified 220 genes (false discovery rate, $FDR \leq 0.05$ and fold change ≥ 1.4) as differentially expressed between E+TN and E+HS rats. Of which, 58 genes were up-regulated and 162 genes were down-regulated in E+HS compared to E+TN (Table 6. 2) rats. Hierarchical clustering was used to group the differentially expressed genes between the treatment groups, based on the level of gene expression. The clustering program divided the rats into two groups, with all the rats from each treatment group clustering together, which suggests less biological variation within the treatment group (Figure 6. 4). Bootstrap testing supported the hierarchical clustering results with 100% confidence. The differentially expressed genes between the treatment groups represented various important pathways such as oxidative phosphorylation, cholesterol biosynthesis, gluconeogenesis, immune response, antioxidant activities and xenobiotic metabolism (Table 6. 3). The functions of six differentially expressed genes are unknown (data not shown). The list of differentially expressed genes with their biological significance is shown in Table 6. 2.

Real-time PCR results

Quantitative real-time PCR was used to confirm the validity of the microarray results. All the eight, randomly selected genes had a similar expression pattern as observed in microarray results, thereby validating the microarray results (Figure 6. 8).

CYP3A1 protein expression and antioxidant enzyme activities and apoptosis rates

Fixed liver sections stained for CYP3A1 primary antibody revealed its greater protein expression in E+HS compared to E+TN rats (Figure 6. 5). There were no histological differences between E+HS and E+TN livers, except for the loss of glycogen and fat energy reserves in E+HS livers (Figure 6. 5). The loss of lipid and glycogen droplets in E+HS hepatic tissue can be attributed to the reduced caloric intake of rats under this condition. Activities of the antioxidant enzymes SOD, and GPx, were reduced in E+HS compared to E+TN rats, with no difference in catalase activities (Figure 6. 6). Number of hepatic cell nuclei that were positive for the TUNEL assay were greater ($p = 0.03$) in E+HS compared to E+TN rats (Figure 6. 7).

5. DISCUSSION

Physiological responses to fescue toxicosis

The E+ diet intake under TN conditions did not change core temperature in both treatment groups. Osborn et al. (1992) reported similar results with no difference in rectal temperature in steers fed an E+ (seed) diet under TN conditions. Exposure to HS increased average daily Tc of E+ rats in the present study. These results are in agreement with previously published results in rats (Spiers et al., 2005a), sheep (Hemken et al., 1979) and steers (Osborn et al., 1992), and impaired animal's ability to dissipate core temperature due to peripheral vasoconstriction (Aldrich et al. 1993) could be the main

reason for elevated Tc under HS conditions. Core temperature of E+TN rats did not change from pre-heat level during this period.

Feed intake of E+TN and E+HS rats decreased on Day 1 from pretreatment level as a result of an E+ diet intake. These results are in agreement with previously published reports in rats (Roberts et al., 2002; Spiers et al., 2005a; Settivari et al., 2006) and steers (Osborn et al., 1992). The signs of adaptation observed in E+TN and E+HS rats with an increase in FI from Day 1 level are in agreement with earlier reports (Settivari et al., 2006). The FI was similar between E+HS and E+TN rats during this period since both groups were maintained under similar environmental conditions and fed same diet during this period. Heat stress exposure decreased FI in E+HS rats within the first 24 hours. Similar reduction in FI was reported in E+ rats maintained under HS conditions (Spiers et al., 2005a; Al-Tamimi et al., 2007). By the end of Day 8, FI of E+HS rats returned to pre-heat level suggesting partial adaptation; however the values were much depressed compared to pre-treatment level. Feed intake of E+TN rats did not change from pre-heat level during this period.

Feed conversion efficiency was decreased in both groups within 24 hours of an E+ diet intake under TN conditions. Settivari et al. (2006) observed similar decrease in FCE of E+ rats maintained under similar environmental conditions. Recovery observed in FCE of both groups by Day 2 suggest adaptation of rats to E+ diet under TN conditions and these results are in agreement with previous published results (Settivari et al., 2006). Heat stress exposure decreased FCE of E+HS rats within the first 24 hours and has previously been reported to reduce FCE of rats (Neal and Schmidt, 1985; Al-Tamimi et al., 2007) as well as in steers (Schmidt et al., 1982; Hoveland, 1993). Reduced FCE could

hinder the ability of E+HS rats to utilize and convert feed energy into productive aspects, often observed in fescue toxicosis. The toxic effect of ergovaline at the level of gastrointestinal tract epithelium partially explains the suppressed FCE in E+ rats.

Water intake did not differ during TN period between the two groups, however during HS, the values for E+HS rats increased within the first 24 hours. Increased water intake of E+HS rats in the present study was in synchrony with HS, and is in agreement with Al-Tamimi et al. (2007). The increase in water intake is required to maintain water balance and avoid dehydration of rats under HS conditions (Al-Tamimi et al., 2007).

Decreased weight gain is considered to be one of the classical signs of fescue toxicosis. The E+ diet intake, in the present study, decreased growth rate in both groups under TN conditions. These reports are in agreement with previously published observations in rats under TN conditions (Settivari et al., 2006). Heat stress decreased growth rate in E+HS rats from pre-heat level and is in accordance with earlier reports (Spiers et al., 2005a; Al-Tamimi et al., 2007). Growth rate of E+TN rats during this period exhibited adaptation and their values were similar to pre-treatment level. Decreased growth rate in E+ rats under TN and HS conditions could be attributed to decreased FI and FCE.

Observed reduction in average wet liver weights of E+HS rat in the present study could be due in part, to reduced caloric intake and is also similar to previous reports (Settivari et al., 2006). The reduced lipid and glycogen stores in E+HS hepatic tissue (Figure 6. 5) further support this hypothesis. Although intake of an E+ diet reduces serum prolactin levels (Cross et al., 1995), heat stress increases its concentration in rats

(Siegel et al., 1979) and ewes (Hooley et al., 1979). Therefore the observed increased serum prolactin levels in the present study could be due to the direct effects of heat stress.

Genomic responses to fescue toxicosis

Short-term intake of an E+ diet at TN followed by an acute heat stress was hypothesized to result in changes in liver gene expression representing characteristic pathophysiology associated with fescue toxicosis. Microarray and real-time PCR results demonstrated that the expression of genes coding for specific physiological pathways, including oxidative phosphorylation, immune function, xenobiotic metabolism, lipid and protein transport, and chaperone activity were altered by the combination of E+ diet and heat when compared to E+ diet alone at TN. The altered genomic responses observed in the present study could be due to the direct effects of endophytic toxins or heat stress or their combination. Secondly, some of the noted changes could be due to decreased caloric intake. Furthermore, the genomic changes in E+HS rats could be either additive or synergistic effects of E+ toxins with heat stress. These genomic effects and their relationship to the observed physiological responses are expected to provide additional insight into the pathophysiology of fescue toxicosis under HS.

Oxidative phosphorylation and energy metabolism

Genes associated with oxidative phosphorylation and ATP production was down-regulated in E+HS compared to E+TN rat livers. Genes, such as NADH dehydrogenase 1 α , cytochrome C oxidase Va, cytochrome C oxidase VIc, pyruvate-ferredoxin oxidoreductase, alanine dehydrogenase, and cytochrome P450 oxidoreductase, play a major role in electron transport (NIH-DAVID; Dennis et al., 2003). Therefore, down-regulation of these genes in E+HS vs. E+TN rats would increase impairment of oxidative

phosphorylation as well as electron leak, resulting in formation of superoxide radicals. Furthermore, down-regulation of genes associated with ATP production, such as ATPase H^+ transporting and ATP synthase, could reduce energy utilization (NIH-DAVID; Dennis et al., 2003). Settivari et al. (2006) observed down-regulation of various genes associated with oxidative phosphorylation and ATP synthesis in E+ compared to E- rats at thermoneutral conditions. Therefore, it can be speculated that under HS, oxidative phosphorylation and ATP synthesis pathways are more severely affected in E+ fed animals.

Optimum concentration of cellular sodium and potassium are essential for maintenance of cell potential and regulation of cellular volume. Sodium export from cell provides the driving force for several facilitated transports, which import glucose, amino acids and other nutrients into cell (NIH-DAVID; Dennis et al., 2003). The intracellular Na and K concentrations are maintained by Na^+/K^+ -ATPase (Konobasova et al., 1985). Also, carnitine palmitoyl transferase 1a (CPT1A) transports long chain fatty acids into mitochondria for energy release especially when the hepatic glycogen is depleted during starvation (Shin et al., 2006). Down-regulation of Na^+/K^+ -ATPase and CPT1A genes in E+HS compared to E+TN rats might further result in impaired energy utilization. Various genes associated with nutrient transportation are down-regulated in E+ compared to E- rats at TN (Settivari et al., 2006). Down-regulation of various transporter genes in E+HS in the present study compared to E+TN rats suggest greater impact of E+ diet under HS conditions. Impaired energy utilization was previously reported in calves exposed to HS alone (Baccari et al., 1983) or E+ fed rats (Neal and Schmidt, 1985) under HS condition. To compensate for the reduced caloric intake and energy utilization, genes

associated with carbohydrate, lipid and protein metabolism were up-regulated to turn on the gluconeogenesis pathways (Table 6. 2). However, under TN genes associated with lipid and carbohydrate metabolism were down-regulated, but protein catabolism was up-regulated in E+ versus E- rats. Greater reduction in feed intake and FCE in E+ fed rats during HS might explain the reason for up regulation of various gluconeogenic genes in the present study.

Serum lipid and cholesterol levels

Various genes associated with lipogenesis, such as stearyl-coenzyme A desaturase, thyroid hormone-responsive spot 14, thyroid hormone-responsive spot 14 homolog, lipogenic protein S14, putative delta 6-fatty acid desaturase, fatty acid desaturase 1, arylformamidase, triose-phosphate isomerase, isopentynyl-diphosphate delta isomerase 1, acetyl co A acetyltransferase 2, and adipose differentiation related protein were down-regulated in E+HS compared to E+TN rats. Similarly, many genes coding for cholesterol biosynthesis, such as NADPH-dependent butanol dehydrogenase, Cyp51, protein kinase AMP activated beta 1 non-catalytic subunit, insulin induced gene 1, isopentynyl diphosphate delta isomerase, NADPH steroid dehydrogenase-like protein, IPP isomerase, and 24-dehydroxy cholesterol reductase were down-regulated in E+HS compared to E+TN rats. Vimentin transports majority of the cholesterol into mitochondria, where it is converted to steroids (NIH-DAVID; Dennis et al., 2003). Down-regulation of vimentin in E+HS vs. E+TN rats could result in impaired steroidogenesis. In addition, many other genes associated with steroidogenesis such as RAR-related orphan receptor c, ald protein, and t-complex protein 1 were also down-regulated in E+HS compared to E+TN rats. Cytochrome P450 8B1 plays a major role in

cholic acid biosynthesis from cholesterol and its expression is inversely proportional to serum cholesterol levels (Chen et al., 2005). Up-regulation of this gene in E+HS versus E+TN further suggests a possible mechanism for reduction of serum cholesterol levels in E+HS rats. Cytochrome P450 7A1 is a rate-limiting enzyme for bile acid synthesis from cholesterol (Chen et al., 2005). Down-regulation of this gene in E+HS versus E+TN could impair the ability to absorb dietary lipids in the intestine. Reduced serum cholesterol and total lipid levels have been reported in cattle grazing on E+ pasture under HS conditions (Stuedemann et al., 1985). Intake of an E+ diet at TN down-regulates various genes associated with sex-steroid metabolism, cholesterol, and lipid metabolism in mice (Bhusari et al., 2006) and rats (Settivari et al., 2006). Down-regulation of these pathways in E+HS in the present study suggest greater impact of fescue toxicants on cholesterol and steroid biosynthesis under HS, which again support the well-characterized clinical sign of fescue toxicosis (i.e., reduced male and female reproductive efficiency).

Immune response

Many genes associated with immune function, such as haptoglobin, conserved helix-loop-helix ubiquitous kinase, protein tyrosine phosphatase 4a1, ccaat/enhancer binding protein beta, cyclophilin, rap guanine nucleotide exchange factor, fc fragment of igg receptor transporter alpha, fc receptor igg alpha chain transporter, neonatal fc receptor, macrophage migration inhibitor factor, and il2-inducible t-cell kinase were down-regulated in E+HS compared to E+TN rats. Similar reduction in genes coding for immune system was reported earlier in E+ versus E- rats under TN conditions (Settivari et al., 2006). Present results suggest a further reduction in the immune system efficiency

in E+ fed rats under HS conditions. Decreased immune response, red and white blood cell counts, response to mitogens, and increased T-suppressor cell number in the spleen were previously observed in fescue toxicosis (Dew et al., 1999; Filipov et al., 1999). Greater susceptibility of E+ mice to subcutaneous injection of cytokines, and less tolerance of cattle grazing on E+ pastures to the effects of lipopolysaccharides (Filipov et al., 1999a, b) further suggest that fescue toxicants make animals more susceptible to various stressors.

Detoxification and antioxidant activity

Primary hepatic detoxification processes include xenobiotic biotransformation (phase I metabolism) and the subsequent conjugation of the resulting metabolites (phase II metabolism), making them more water soluble and available for excretion from the body. Phase I metabolism mainly involves the cytochrome P450 (CYP) and flavin monooxygenase enzymes. Cytochrome P450 2B10, which is orthologous to human CYP2B6, is known to metabolize various drugs and xenobiotics (Gnerre et al., 2005). Similarly, CYP2J13 is orthologous to human CYP2J2 and is known to be actively involved in xenobiotic metabolism (Yamazaki et al., 2006). The flavin-containing monooxygenase gene catalyzes the NADPH and oxygen-dependent oxidation of several drugs and xenobiotics (Ziegler, 2002). Down-regulation of this gene further hampers E+HS rat's ability for detoxification. Other genes associated with xenobiotic metabolism include CYP51, CYP2C70, and alcohol dehydrogenase. All these genes were down-regulated in E+HS compared to E+TN rats, to potentially impair xenobiotic metabolism. In contrast to the present findings, E+ fed rats under TN condition (Settivari et al., 2006) had greater gene expression for various CYP isoforms compared to controls.

Hepatic protein expression of CYP3A1 was greater in E+HS versus E+TN (Figure 6. 5) in the present study. Bhusari et al. (2006) observed a similar increase in CYP3A expression in E+HS mice compared to E+TN mice. Moubarak et al. (2000) observed hydroxylation of ergotamine (a constituent of fescue toxins) by CYP3A present in beef liver microsomes. In another study, Moubarak et al. (2003b) induced CYP3A levels in rat liver microsomes and then treated the preparation with ergotamine. This produced a greater metabolism of ergotamine than in controls due to CYP3A-induced microsomes, and resulted in production of dihydroxy derivatives. Peyronneau et al. (1994) reported metabolism of bromocriptine, an ergot alkaloid similar to ergotamine, by CYP3A in rat liver microsomes treated with dexamethasone, a CYP3A inducer. They also reported that ergopeptides, like bromocriptine, ergocryptine, and dihydro-ergotamine, exhibit high affinity for rat CYP3A. Althus et al. (2000) observed the metabolism of another ergot alkaloid, alpha-dihydroergocryptine, by CYP3A isoforms. Therefore, it can be speculated that up-regulation of CYP3A1 in E+HS would result in greater metabolism of ergot alkaloids associated with fescue toxicosis. Differences in the expression pattern of various CYP isoforms, in the present study, suggest the complexity associated with the effects of E+ diet on different CYP isoforms.

The liver is one of the major sites for heme synthesis, and 2/3rd of the heme synthesized in liver is utilized for the formation of CYP (Fujita, 1997). However, many genes associated with heme biosynthesis such as transferrin, transferrin receptor, aminolevulinic acid synthase (rate limiting enzyme for heme biosynthesis), uroporphyrinogen decarboxylase, and kallikrein b were down-regulated in E+HS compared to E+TN rat hepatic tissue. Down-regulation of various genes associated with

heme biosynthesis could, at least in part, explain the reason for reduced CYP gene expression in E+HS rats.

Gamma glutamate-cysteine ligase (GCL), a rate-limiting enzyme in glutathione (GSH) synthesis, was down-regulated in E+HS rats in the present study. Glutathione, which constitutes up to 10% of cytosolic proteins in mammalian cells, is also an important free radical scavenger in the cell (DuTeaux et al., 2004). Therefore, down-regulation of GCL in E+HS compared to E+TN rats could hamper the rat's ability for glutathionation, thereby altering its redox status. Other important enzymes which play a major role in hepatic phase II detoxification and free radical neutralization, such as epoxide hydrolases (Ephx), hydroxyl glutathione hydrolase, and triosephosphate isomerase, superoxide dismutase and stearyl-coenzyme A desaturase I, were down-regulated in E+HS compared to E+TN rats. Down-regulation of the above mentioned genes in E+HS rats would result in reduced ability of the rats to neutralize various free radicals, such as superoxide radicals, produced as a result of electron leak during oxidative phosphorylation.

One of the primary enzymatic defenses against reactive oxygen species is SOD, which catalyses the conversion of superoxide anions into H_2O_2 (Scandalios, 1997). Enzyme activity of SOD was reduced in E+HS versus E+TN rats in the present study (Figure 6.6), making them more susceptible to superoxide radical associated oxidative stress. About 50% reduction in SOD results in a functional decline of oxidative phosphorylation and an increase in oxidative stress apoptosis rates (Wheeler et al., 2001). Another important antioxidant enzyme in cell, GPx converts harmful H_2O_2 into water and molecular oxygen (Liska, 1998). In the present study, decreased enzyme activity of GPx

in E+HS rats could hamper the rat's ability to neutralize H₂O₂. The H₂O₂ undergo hemolytic scission on the inner mitochondrial membrane and mitochondrial matrix causing site-specific damage of critical matrix targets. Its decomposition outside the mitochondria may cause damage to the outer mitochondrial membrane itself and /or cytosolic targets, within the diffusion limit of hydroxyl radical (Liska, 1998). It is anticipated that cells containing low levels of one or more of these enzymes would be particularly vulnerable to oxidative damage (Liska, 1998; Tiedge et al., 1998). Lakritz et al. (2002) reported reduced blood glutathione levels in E+ fed cattle, compared to controls, during heat stress. Gorini et al. (1988) observed a reduction in forebrain glutathione levels in male rats after repeated ip injection of dihydroergocristine and dihydroergocryptine. Earlier reports suggest that rats fed with an E+ diet at TN exhibit a similar reduction in gene expression of various phase II enzymes and antioxidants (Settivari et al., 2006). The current findings imply that E+ diet fed rats experience greater oxidative stress and impaired detoxification processes under HS than at TN conditions. In contrast to the present findings, Bhusari et al. (2007) observed an up-regulation of various antioxidants in E+ diet fed mice at the end of two week HS exposure. Differences in the species and exposure time could be the reasons for the observed alterations for the effects of E+ on antioxidant enzymes.

Cell proliferation and apoptosis

Intake of an E+ diet during HS down-regulated various genes associated with apoptosis inhibition, including apoptosis inhibitor 5, neuronal apoptosis inhibitory protein related sequence 4, and prothymosin alpha. Also, the genes associated with apoptosis, such as amyloid beta precursor protein binding protein 1, notch gene homolog 2,

peptidyl-prolyl cis-trans isomerase a, and ring finger protein 125 were up-regulated in E+HS compared to E+TN rats. Earlier reports suggest similar hepatic gene expression pattern in E+ diet fed rats, even under TN conditions (Settivari et al., 2006). Therefore, the present study implies that intake of the E+ diet during HS produces a greater hepatocyte apoptosis rate. Increased TUNEL positive hepatocytes, observed with TUNEL assay, further validated the current microarray results (Figure 6. 7). A combination of E+ toxins, heat stress, and reduced caloric intake could have contributed to the increased TUNEL positive hepatic nuclei in the E+HS group.

Cell proliferation is a representation of cell growth and active division. Fescue toxicants, in the present study, down-regulated many genes associated with cell proliferation, including microtubule-associated protein rp/eb family member 2, ornithine decarboxylase, phosphoinositide-3-kinase regulatory subunit, beta actin, glycogen synthase kinase 3 beta, protein phosphatase 2 a catalytic subunit alpha, cyclin D1, cyclin A2, cyclin L2, tyrosine protein kinase, go/g1 switch gene 2, eukaryotic translation initiation factor 4a1, lim and senescent cell antigen-like domain 1, and eukaryotic translation initiation factor 4a in E+HS compared to E+TN rats. Down-regulation of these genes could contribute to reduce cell proliferation rates in E+ diet fed rats under HS.

Chaperones

Chaperones, such as heat shock proteins, are up-regulated in response to a wide variety of stressors, including oxidative stress, starvation, hyperthermia, and exercise (Marshall et al., 2006). The E+HS rats in the present study were exposed to three different stressors i.e., E+ toxicants, heat stress, and reduced caloric intake; and therefore the chaperone activity in this group was expected to be greater compared to E+TN rats.

However, genes coding for chaperone activity such as peptidyl-prolyl cis trans isomerase a, peptidyl prolyl isomerase a, homocysteine-inducible endoplasmic reticulum stress inducible ubiquitin like domain member 1, heat shock protein 70 kda protein 8 (Hspa8), heat shock protein 90 kda protein 1 beta, heat shock protein 105 (Hsp105), chaperone containing tcp1 subunit 5, dnaj (hsp40) homolog subfamily b1, dnaj (hsp40) homolog subfamily b6, dnaj (hsp40) homolog subfamily b11, dnaj (hsp40) homolog subfamily a2, and rotamase were down-regulated in E+HS compared to E+TN rats. Real-time PCR further confirmed the down-regulation of Hspa8 and Hsp105 in E+HS vs. E+TN rats (Figure 6. 8). In stressed cells, heat shock factor 1 (HSF1) mediates the induction of heat shock protein gene expression. In response to stress, HSF1 undergoes stress-induced modifications at lysine 298 by the small ubiquitin-like modifier 1 (SUMO-1). Upon this modification, HSF1 acquires DNA binding ability and localizes to nuclear stress granules, to drive the expression of various HSPs (Hong et al., 2001). In the present study, gene expression of HSF1 and SUMO-1 was measured using real-time PCR. Gene expression of HSF1 was similar ($p = 0.95$) between E+HS (dCT value = 1.58) and E+TN (dCT value = 1.57) rat livers; however the expression for SUMO-1 was significantly ($p = 0.01$) reduced in E+HS vs. E+TN rats (Figure 6. 8). Therefore, down-regulation of SUMO-1 could hamper HSF1's DNA binding activity and thereby HSP transcriptional activation. Settivari et al. (2006) reported similar reduction in hepatic chaperone gene expression in E+ fed rats compared to E- controls under TN conditions. Present findings suggest greater reduction in gene expression of various chaperones in E+ diet fed rats under HS than at TN conditions. Other unidentified mechanisms may also contribute to these

unexpected results and further studies are required in this area for better understanding of the mechanisms involved.

E-HS vs E+HS

Microarray analysis between E- and E+ rats under HS conditions, identified 11 genes as differentially expressed of which, five were up-regulated and six genes were down-regulated in E+HS rats. Of the differentially expressed, genes coding for antioxidant as well as glucose and lipid metabolism were down-regulated and CYP2C37 was up-regulated in E+HS rats. Since the number of differentially expressed genes representing each pathway were very few, it is hard to draw conclusions for this comparison. One possible reason for less genomic variation between these two groups could be the high biological variation observed within each treatment group, which is evident in the hierarchical map (Figure 6. 9).

6. CONCLUSION

The current study provides information regarding specific hepatic genes, which are differentially expressed in E+ rats when exposed to short-term heat stress. The cellular mechanisms involved in the pathogenesis of fescue toxicosis under heat stress conditions were elucidated. Of particular interest were the pathophysiological roles of reduced energy utilization, electron leak resulting in oxidative stress, and impaired detoxification, immune and chaperone functions, making animals more susceptible to

various stressors. Likewise, the development of genomic signs of fescue toxicosis during heat stress was important.

Intake of E+ diet at TN conditions resulted in up-regulation of genes associated with CYPs, but down-regulated genes associated with chaperone, antioxidant and immune systems (Settivari et al., 2006). The present study suggested that added effects of HS on E+ intake further impaired the animal's defense mechanisms (chaperone, antioxidant and immune systems) beyond the effect at thermoneutrality, thereby making them more susceptible to various stressors. This might explain why HS exacerbates the clinical signs associated with fescue toxicosis, apart from hyperthermia associated with peripheral vasoconstriction. The present study emphasizes the importance of the inclusion of pair-fed treatment groups in future studies to differentiate the direct hepatic effects of E+ toxins from the secondary effects on the liver that are associated with reduced caloric intake. Future studies should be designed to identify differentially expressed genes after exposure to E+ toxicants under chronic heat stress conditions, to determine specific markers that might be targeted for prophylactic and therapeutic interventions.

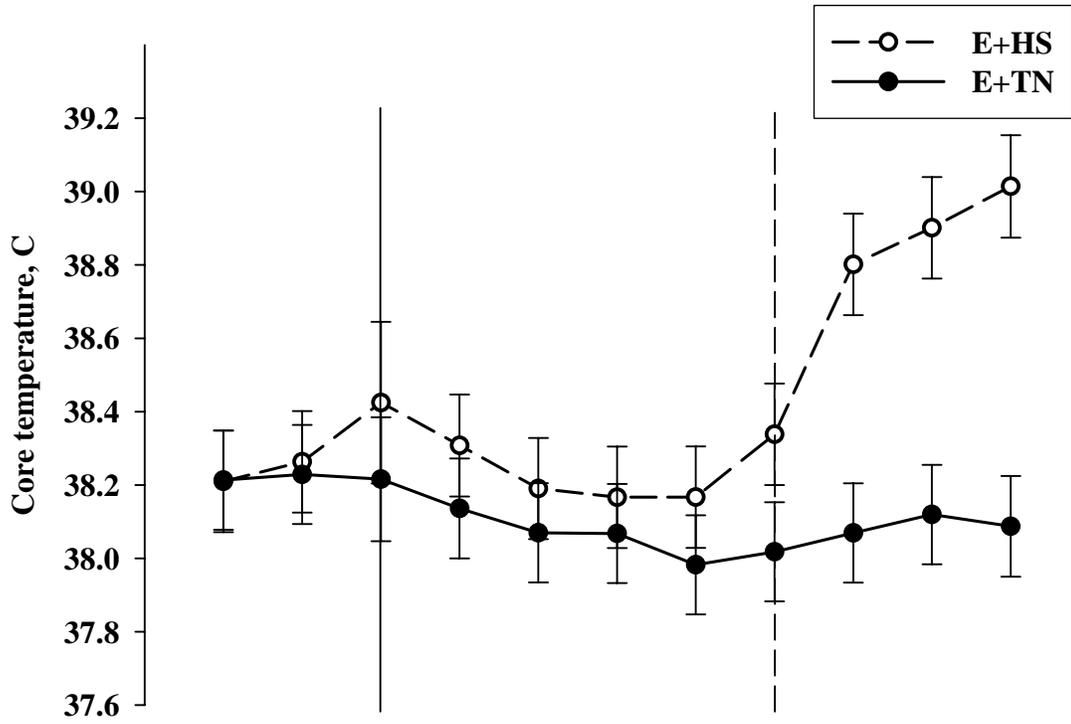


Figure 6. 1a.

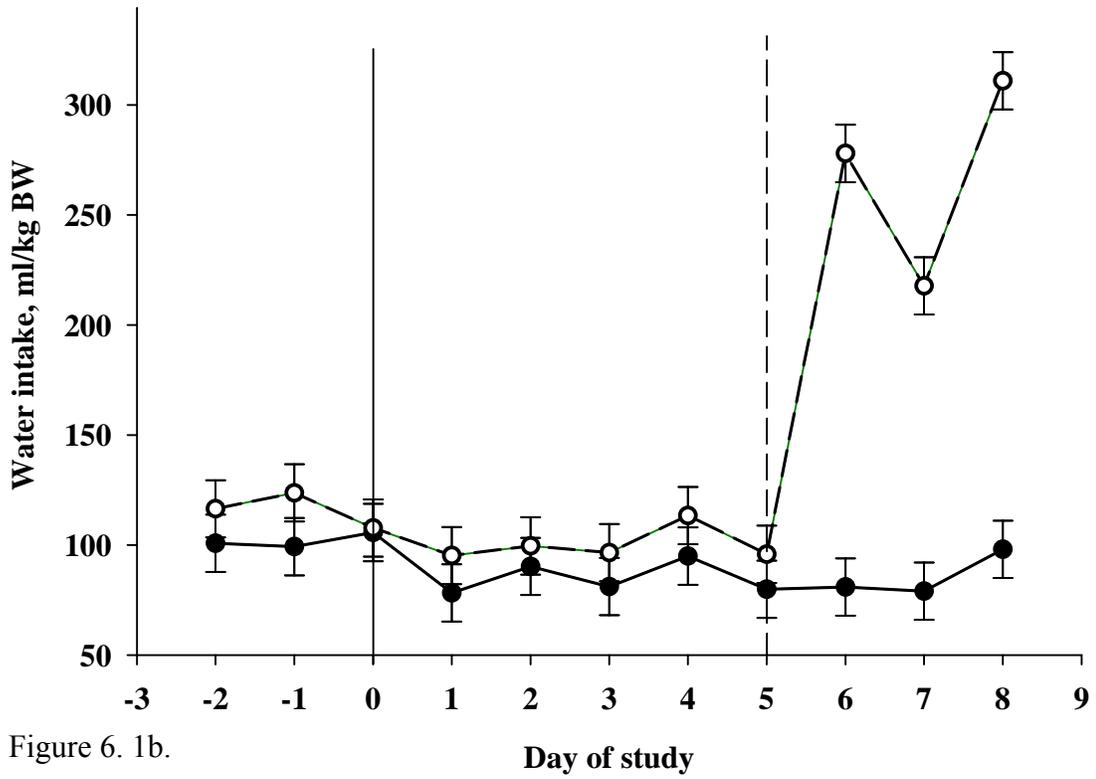


Figure 6. 1b.

Figure 6. 1a. Average core body temperature of rats fed E+ diet and maintained under TN (21°C) for five days, and then extended under TN or HS conditions (31°C) for three days. Core temperature for E+HS was greater ($p < 0.05$) during HS period from E+TN level.

Figure 6. 1b. Average water intake of rats fed E+ diet and maintained under TN (21°C) for five days, and then extended under TN or HS conditions (31°C) for three days. Water intake for E+HS was greater ($p < 0.05$) during HS period from E+TN level.

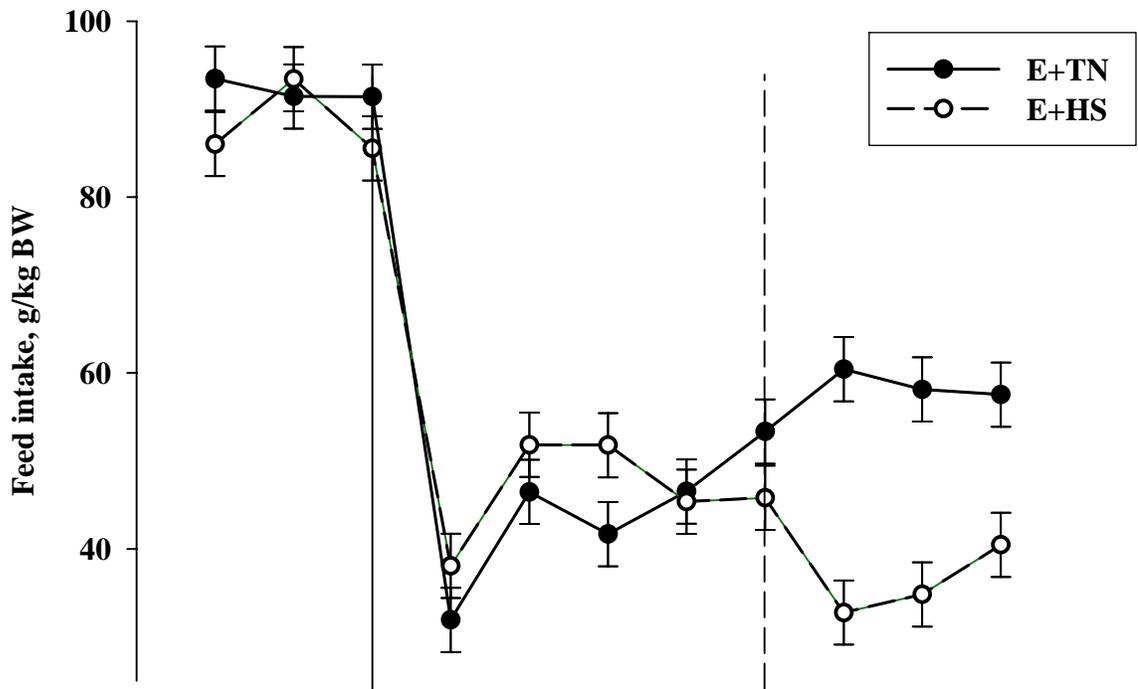


Figure 6. 2a.

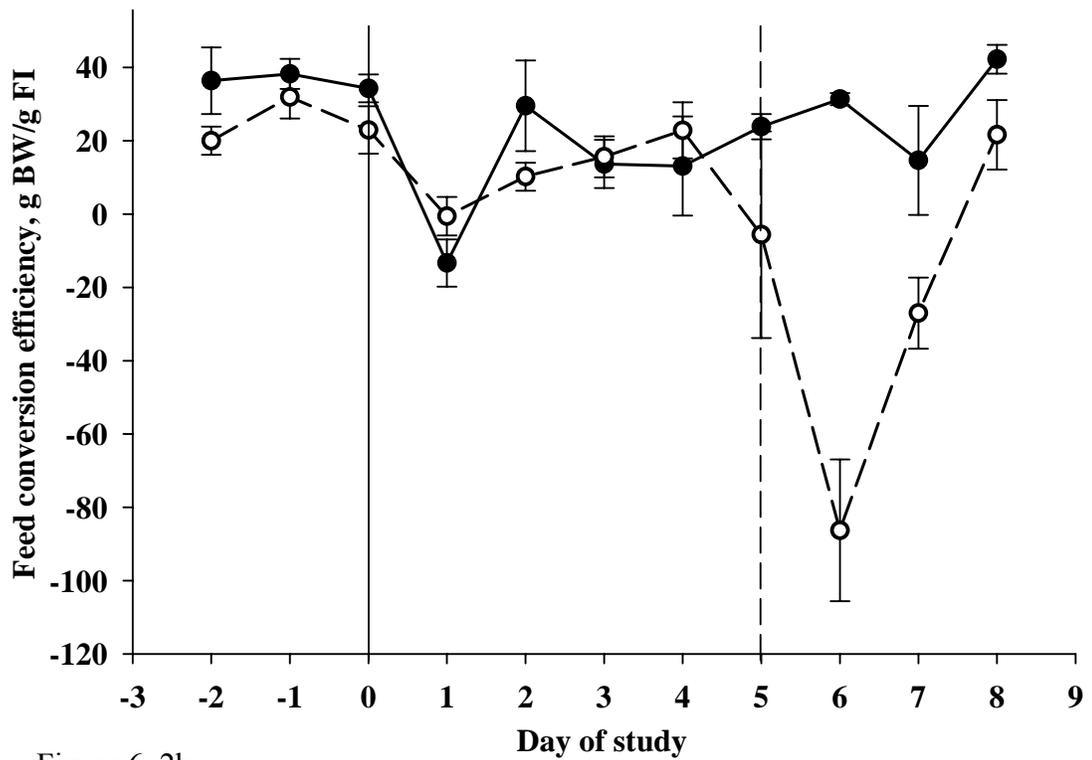


Figure 6. 2b.

- Figure 6. 2a. Fescue toxicosis-induced reduction in average daily feed intake during the eight-day treatment period for E+TN and E+HS groups. Feed intake for E+HS was significantly reduced ($p < 0.05$) from E+TN during HS period.
- Figure 6. 2b. Fescue toxicosis-induced reduction in average feed conversion efficiency during the eight-day treatment period for E+TN and E+HS groups. Feed conversion efficiency for E+HS was significantly reduced ($p < 0.05$) from E+TN during HS period.

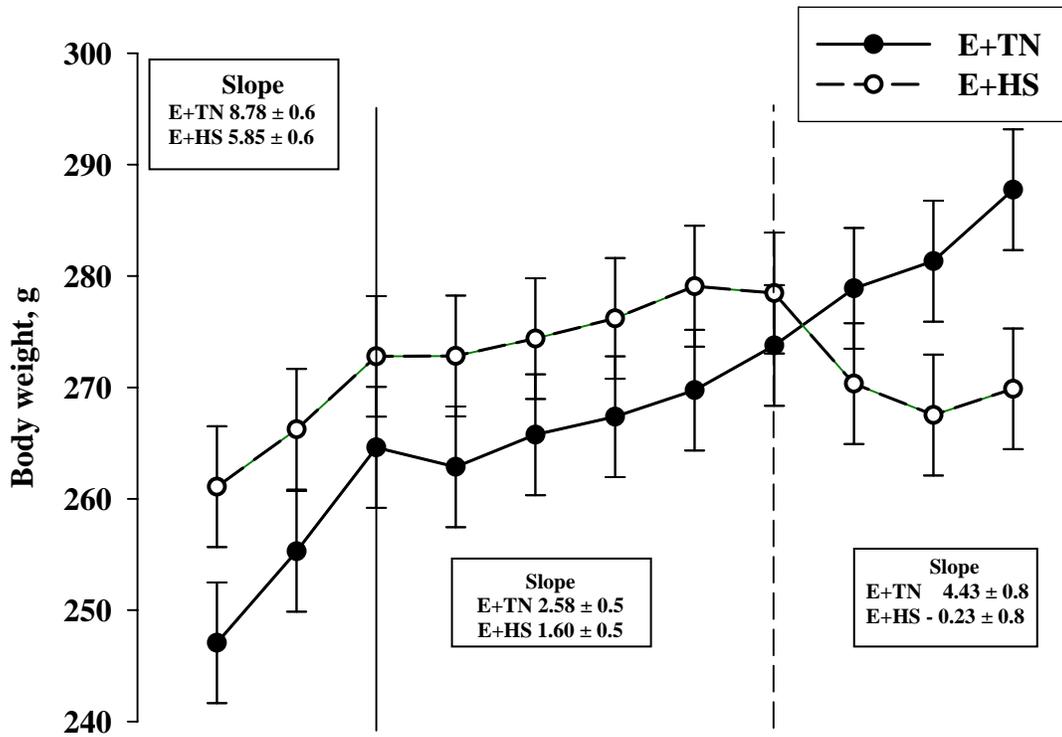


Figure 6. 3a.

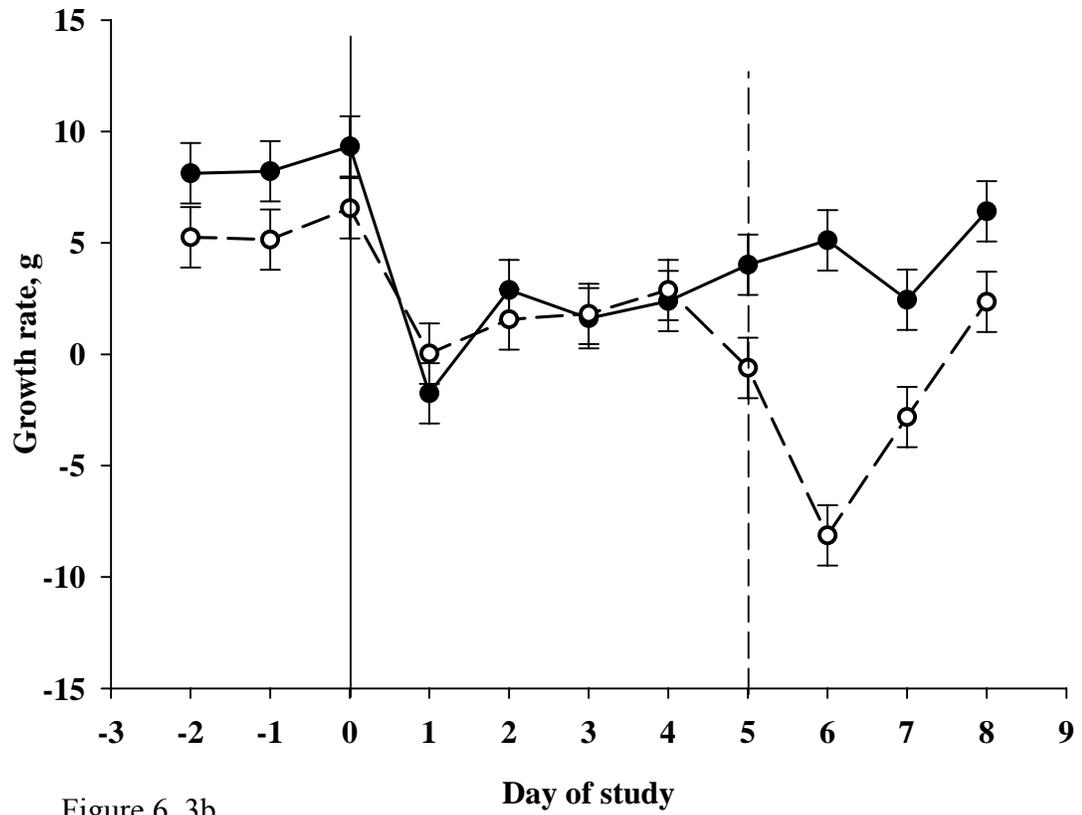


Figure 6. 3b.

Figure 6. 3a. Fescue toxicosis-induced reduction in average body weight during the eight-day treatment period for E+TN and E+HS groups. Body weight for E+HS was significantly reduced ($p < 0.05$) from E+TN during HS period.

Figure 6. 3b. Fescue toxicosis-induced reduction in average growth rate during the eight-day treatment period for E+TN and E+HS groups. Growth rate for E+HS was significantly reduced ($p < 0.05$) from E+TN during TN and HS periods.

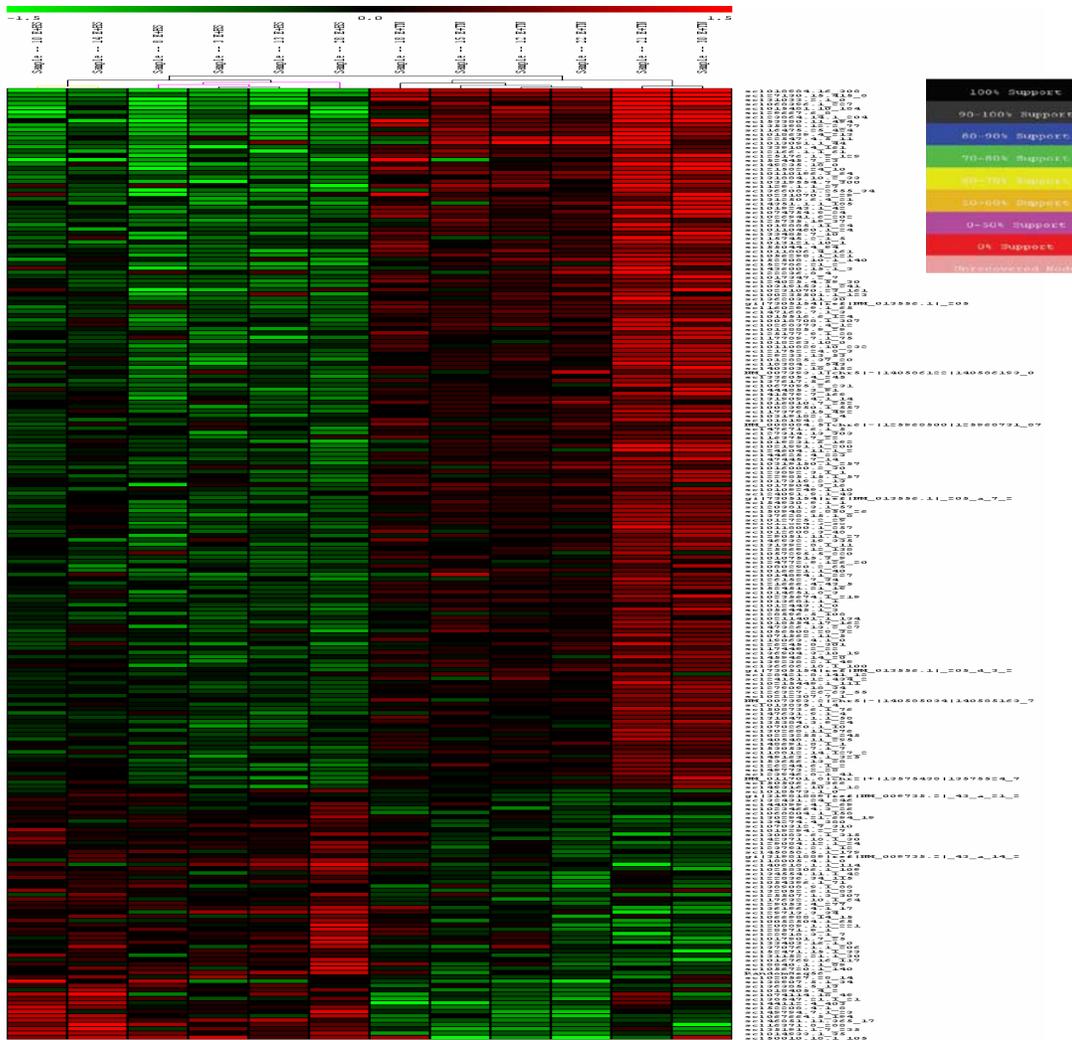


Figure 6. 4. Heat map representing differentially expressed genes in E+HS compared to E+TN rats maintained under thermoneutral (TN) conditions (21°C) for five days, and then extended under TN or heat stress (HS) conditions (31°C) for three days.

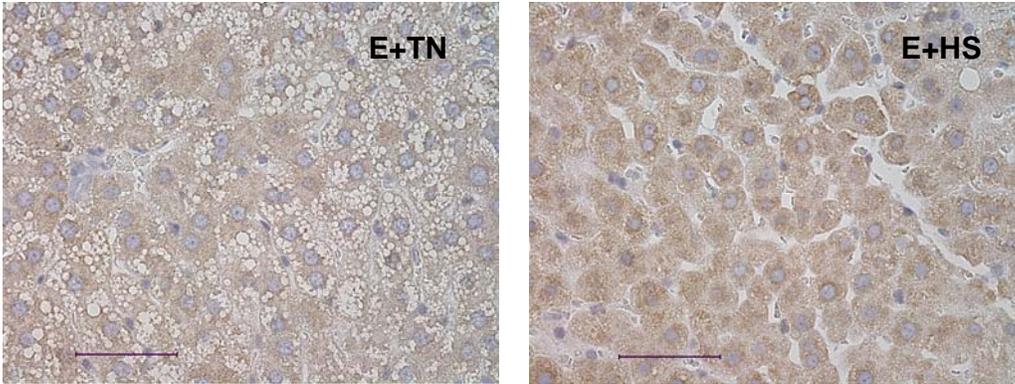


Figure 6. 5. Average protein expression of CYP3A1 for hepatic tissue from E+TN and E+HS groups. This expression was greater ($p < 0.05$) in E+HS compared to E+TN groups.

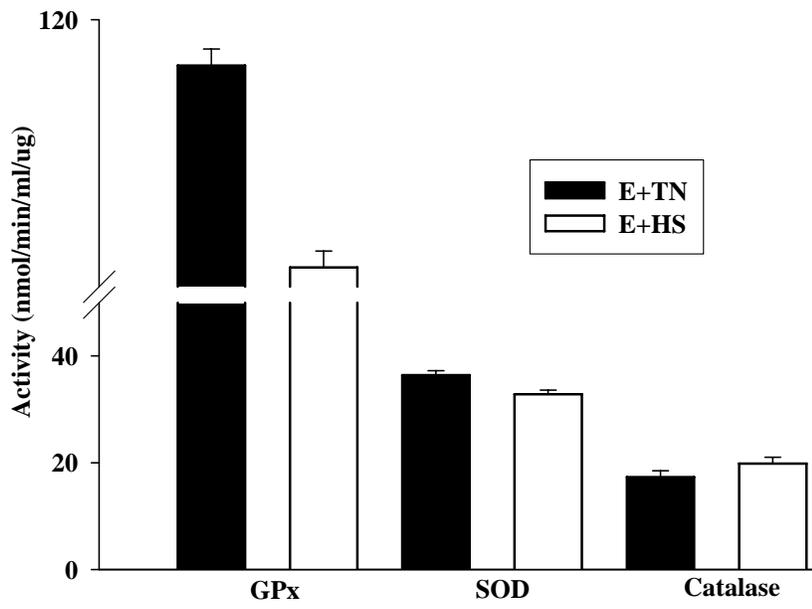


Figure 6. 6. Average antioxidant activities for hepatic tissue from E+TN and E+HS groups. This expression for glutathione peroxidase, copper-zinc superoxide dismutase and catalase were reduced ($p < 0.05$) in the livers of E+HS compared to E+TN groups.

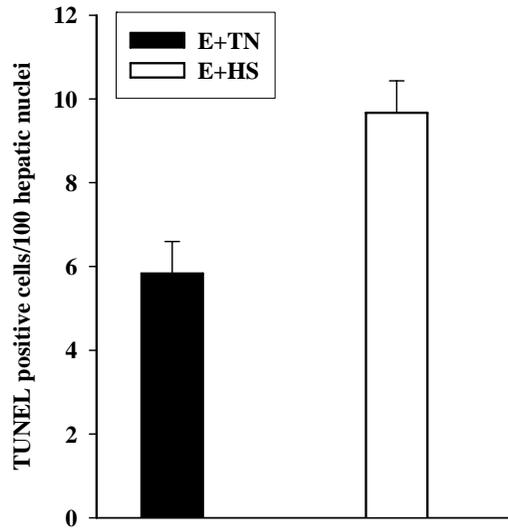


Figure 6. 7. Average number of TUNEL positive cells per 100 hepatic nuclei for E+TN and E+HS groups. The number of TUNEL positive hepatocytes were greater ($p = 0.03$) in the livers of E+HS compared to E+TN groups.

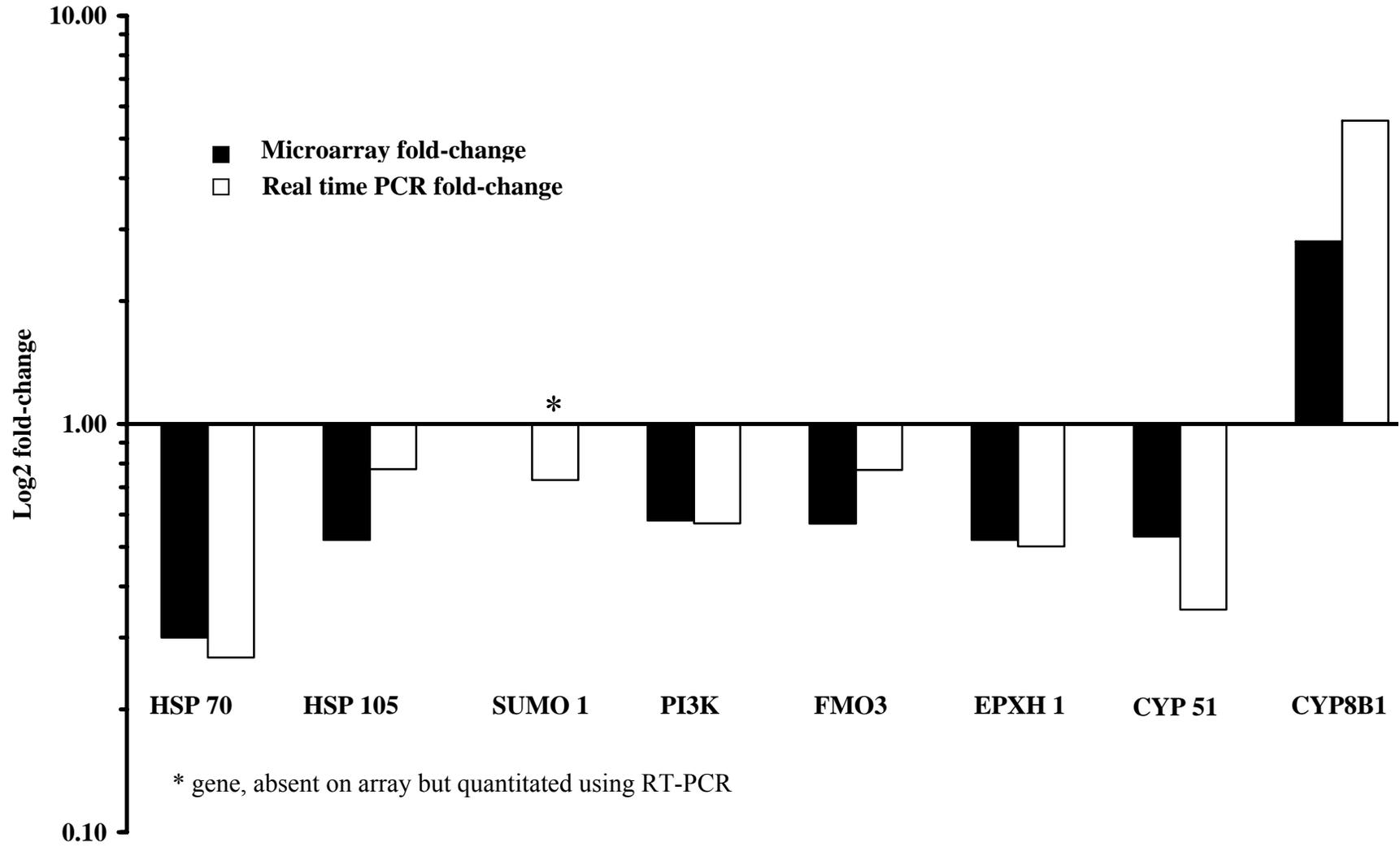


Figure 6. 8. Gene expression for eight genes, randomly selected from the differentially expressed gene list of microarray results. The values are presented as the log₂ ratios computed from the gene expression in E+HS over E+TN groups. Solid bars represent gene expression results observed with microarray experiments, whereas open bars represent the results from real-time PCR. Gene expression for all the above eight genes, was significantly ($p < 0.05$) different between E+HS vs. E+TN rats in both microarray and real-time PCR experiments.

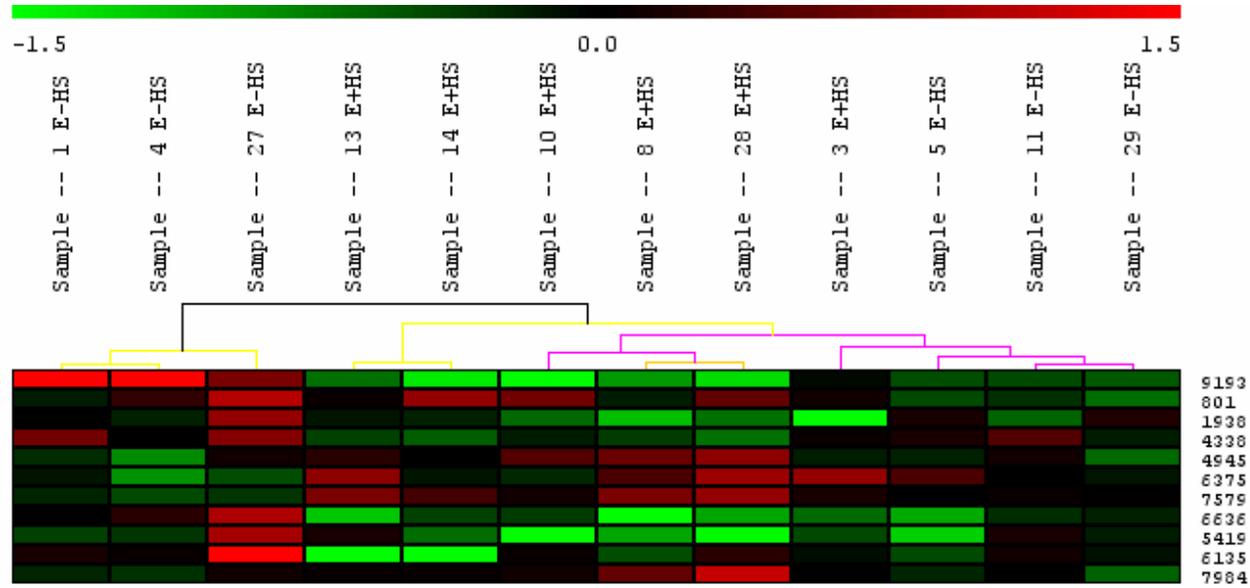


Figure 6.9. Heat map representing differentially expressed genes in E+HS compared to E-HS rats maintained under thermoneutral (TN) conditions (21°C) for five days, and then extended under heat stress (HS) conditions (31°C) for three days.

Table 6. 1. Primer sequences (5' → 3') used in real-time PCR

Name	Symbol	Forward primer	Reverse primer	Product size ^a (bp)
Flavin containing monooxygenase 3	Fmo3	GGACTGTGTTTGAGGGCATT	ACTGCCATGGTTGGTTTCTC	190
Small ubiquitin-like modifier	SUMO1	TTGGACAGGACAGCAGTGAG	CCATTCCCAGTTCTTTTGGGA	168
Heat shock protein 70	Hsp70	CAGAATCCCCAAGATCCAGA	GTGACATCCAAGAGCAGCAA	168
Heat shock factor 1	Hsf1	CCAGCAGCAAAAAGTTGTCA	TGGTGACAGCATCAGAGGAG	239
Phosphoinositide kinase-3	Pi3k	ACCAGTGTTGACCCTTCCTG	GGCGAGATAGCGTTTGAAAG	244
Cytochrome P450 51	CYP51	CCTTCACGCTTAGCCTTGTC	GTGAAAGTCTTGCCACCAT	214
Epoxide hydrolase 1	Ephx1	AAGCCATCAGCCAAAGAAGA	GCTTCCTCCAGTCAAACCTCG	202
Heat shock protein 105	Hsp105	AGTTGCCCACTGGATTAACG	CAAGCAATTGAGACCAGCAA	227
Cytochrome P4508B1	CYP8B1	GGTGGCTCTCTTCCCCTATC	CAGGGCATGTTGTAGTGGTG	156

^aProduct size, bp, includes the sequences of forward and reverse primers

Table 6. 2. Differentially expressed gene list in E+HS compared to E+TN rats at the end of 8 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
<i>Differentially expressed genes associated with mitochondrial functions</i>					
NM_008898	Por	P450 (cytochrome) oxidoreductase	Electron transport	Down, 0.20	0.000
NM_144900	Atp1a1	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	Maintain cell potential and volume	Down, 0.60	2.1x10 ⁻⁴
NM_133668	Slc25a3	Solute carrier family 25 (mitochondrial phosphate carrier, member 3)	Mitochondrial phosphate carrier	Down, 0.61	0.005
NM_025272	Atp6v0e	ATPase, H ⁺ transporting, V0 subunit	ATPase activity	Down, 0.66	0.009
NM_053071	Cox6c	Cytochrome c oxidase, subunit VIc	Electron transport chain	Down, 0.66	0.018
NM_007747	Cox5a	Cytochrome c oxidase, subunit Va	Electron transport chain	Down, 0.69	0.035
NM_153150	Slc25a1	Solute carrier family 25 (mitochondrial citrate carrier), member 1	Mitochondrial phosphate carrier	Down, 0.71	0.013
NM_008810	Pdha1	Pyruvate dehydrogenase E1 alpha 1	Oxidoreductase activity, glycolysis	Down, 0.71	0.034
NM_015760	Nox4	NADPH oxidase 4	Superoxide generating NADPH oxidase activity	Up, 1.40	0.028
NM_008097	Gcdh	Glutaryl-Coenzyme A dehydrogenase	Glutaryl-CoA dehydrogenase activity, electron transport	Up, 1.49	0.021
NM_053083	Lox14	Lysyl oxidase-like 4	Oxidoreductase activity	Up, 1.59	6.9x10 ⁻⁴
NM_017462	Polg	Polymerase (DNA directed), gamma	Mitochondrial DNA replication	Up, 1.59	0.013
<i>Differentially expressed genes associated with apoptosis</i>					
NM_145564	Fbxo21	F-box only protein 21	Ubiquitination	Down, 0.63	0.001
NM_007466	Api5	Apoptosis inhibitor 5	Inhibits apoptosis	Down, 0.66	0.042
NM_144800	Mtss1	Metastasis suppressor protein 1	Suppress metastasis	Down, 0.57	0.000
NM_008794	Pcsk7	Proprotein convertase subtilisin/kexin 7	Ubiquitous endoprotease activity	Down, 0.68	0.003
NM_144931	Appbp1	Amyloid beta precursor protein binding protein 1	Apoptosis through deregulation of NEDD8 conjugation	Up, 1.4	0.051
NM_010928	Notch2	Notch gene homolog 2	Regulate programmed cell death	Up, 1.5	0.002

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
NM_026301	Rnf125	Ring finger protein 125	Ubiquitination and proteasomal degradation of target protein	Up, 2.1	0.001
<i>Differentially expressed genes associated with blood</i>					
NM_020559	Alas1	Amino levulinate synthase	Heme metabolism	Down, 0.33	0.000
NM_011638	Tfrc	Transferrin receptor	Erythrocyte development, Iron transport	Down, 0.42	2x10 ⁻⁴
NM_021366	Klf13	Kruppel-like factor 13	Transcription factor of erythroid lineage	Down, 0.49	2x10 ⁻⁴
NM_008455	Klkb1	Kallikrein B, plasma 1	Blood coagulation	Down, 0.67	0.030
NM_024457	Rap1b	RAS related protein 1b	Required for normal platelet function	Down, 0.70	0.030
NM_009478	Urod	uroporphyrinogen decarboxylase	Heme biosynthesis	Down, 0.71	0.023
<i>Differentially expressed genes associated with cell cycle and transcription regulation</i>					
NM_011498	Bhlhb2	Basic helix-loop-helix domain containing, class B2	Transcription, nucleic acid metabolism	Down, 0.32	0.000
NM_008059	G0s2	G0/G1 switch gene 2	Cell cycle progression, oncogene	Down, 0.52	0.016
NM_009828	Ccna2	Cyclin A2	Cell cycle transition from G2 to M phase	Down, 0.55	0.006
NM_021462	Mknk2	MAP kinase-interacting serine/threonine kinase 2	Regulate transcription by phosphorylating EIF4E	Down, 0.55	0.000
NM_013556	Hprt	Hypoxanthine guanine phosphoribosyl transferase	Purine metabolism	Down, 0.57	8.69x10 ⁻⁵
NM_013614	Odc	Ornithine decarboxylase	Polyamine production required for cell division	Down, 0.59	0.001
NM_026148	Lims1	LIM and senescent cell antigen-like domains 1	Cell proliferation, differentiation and survival	Down, 0.60	0.013
NM_007393	Actb	Actin, beta, cytoplasmic	Cell motility	Down, 0.60	0.033
NM_018856	Ccnl2	Cyclin L2 transcript variant 2	Cell division	Down, 0.64	0.035
NM_144958	Eif4a1	Eukaryotic translation initiation factor 4A1	Translation initiation	Down, 0.68	0.011
NM_007631	Ccnd1	Cyclin D1	Cell cycle transition from G1 to S phase	Down, 0.68	0.006
NM_153058	Mapre2	Microtubule-associated protein, RP/EB family, member 2	Spindle function by stabilizing microtubules and anchoring them at centrosomes	Down, 0.70	0.034

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
NM_019411	Ppp2ca	Protein phosphatase 2a, catalytic subunit, alpha isoform	Regulation of progression through cell cycle	Down, 0.62	0.012
NM_007393	Actb	Actin, beta, cytoplasmic	Cell motility, cytoskeleton	Down, 0.60	0.033
NM_023580	Epha1	Eph receptor A1	Cell-cell interaction	Down, 0.71	0.006
NM_016744	Pde1a	Phosphodiesterase 1A, calmodulin-dependent	Purifying metabolism, Has a higher affinity for cGMP than for cAMP	Up, 1.4	0.048
NM_177882	A730012O14Rik	RIKEN cDNA A730012O14 gene	Cell division, nucleic acid metabolism, DNA replication	Up, 1.51	5.0x10 ⁻⁴
<i>Differentially expressed genes associated with chaperone activity</i>					
NM_031165	Hspa8	Heat shock protein 8	Protein folding, assembling/disassembling of protein complexes	Down, 0.30	0.000
NM_013559	Hsp105	Heat shock protein 105	Regulate the substrate binding cycle of Hsp70/Hsc chaperone system	Down, 0.52	0.012
NM_022331	Herpud1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Involved in unfolded protein response pathway, Ubiquitin-like function	Down, 0.52	0.004
NM_008302	Hspcb	Heat shock protein 1, beta	Molecular chaperone	Down, 0.58	6.0x10 ⁻⁴
NM_008907	Ppia	Peptidylprolyl isomerase A	Protein folding	Down, 0.59	0.010
NM_018808	Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1	Chaperone activity	Down, 0.61	0.004
NM_011847	Dnajb6	DnaJ (Hsp40) homolog, subfamily B, member 6	Chaperone activity	Down, 0.63	0.013
NM_133167	Parvb	parvin, beta	Regulate cell adhesion and cytoskeleton organization	Down, 0.64	0.004
NM_019794	Dnaja2	DnaJ (Hsp40) homolog, subfamily A, member 2	Chaperone activity one	Down, 0.68	0.042
NM_007637	Cct5	Chaperonin subunit 5 (epsilon)	Chaperone activity	Down, 0.69	0.023
NM_026400	Dnajb11	DnaJ (Hsp40) homolog, subfamily B, member 11	Chaperone activity	Down, 0.72	0.042
NM_145937	AI463102	EST AI463102	Converts newly synthesized inactive sulfatases to their active form, protein metabolism	Up, 1.55	0.046

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
<i>Differentially expressed genes associated with cholesterol and steroid biosynthesis</i>					
BC054446	Abcd3	ATP-binding cassette, sub-family D (ALD), member 3	Reduction of Abcd3 decrease peroxisomal function and increase oxidative stress	Down, 0.43	0.000
NM_134469	Fdps	Farnesyl diphosphate synthetase	Cholesterol biosynthesis	Down, 0.43	0.000
NM_177960	Idi1	Isopentenyl-diphosphate delta isomerase	Cholesterol biosynthesis	Down,0.45	0.001
NM_153526	Insig1	Insulin induced gene 1	Cholesterol homeostasis, cell growth	Down,0.48	0.006
NM_053272	Dhcr24	24-dehydrocholesterol reductase	Steroid biosynthesis and metabolism	Down,0.50	0.000
NM_011281	Rorc	RAR-related orphan receptor gamma C	Steroid hormone receptor activity	Down,0.52	8.6x10 ⁻⁵
BC031813	Cyp51	Cytochrome P450, 51	Sterol 14-demethylase	Down,0.53	2.6x10 ⁻⁴
NM_053272	Dhcr24	24-dehydrocholesterol reductase	Lipid biosynthesis and metabolism	Down,0.50	0.000
NM_024264	Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1	27-hydroxylation of 5-beta- cholestane-3-alpha, bile acid biosynthesis	Down,0.59	0.005
NM_010941	Nsdhl	NAD(P) dependent steroid dehydrogenase-like	Cholesterol biosynthesis	Down,0.64	0.002
NM_031869	Prkab1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Cholesterol biosynthesis	Down,0.67	0.018
NM_009692	Apoa1	in A-I	Cholesterol efflux from tissues to liver	Down, 0.53	0.003
NC_000008	Cyp7a1	Cytochrome P450, 7A1	Cholesterol and steroid biosynthesis	Down, 0.53	2.6x10 ⁻⁴
NM_011701	Vim	Vimentin	Cholesterol transport to mitochondria for steroidogenesis	Down,0.71	0.046
NM_133748	Insig2	Insulin induced gene 2	Essential element of the feed back inhibition system of cholesterol synthesis	Up,2.73	0.000
NM_010012	Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1	Colic acid synthesis	Up,2.8	7.5x10 ⁻⁵
<i>Differentially expressed genes associated with xenobiotics and endotoxin metabolism</i>					
NM_007409	Adh1	Alcohol dehydrogenase 1	Xenobiotic metabolism, lipid metabolism	Down, 0.34	0.001
NM_009998	Cyp2b10	Cytochrome P450, family 2, b10	Oxidizes steroids, xenobiotics	Down, 0.37	6.1x10 ⁻⁴

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
NM_009127	Scd1	Stearoyl-Coenzyme A desaturase 1	Oxygen and reactive oxygen species metabolism, superoxide metabolism	Down, 0.42	0.048
NC_005112	Ephx1	Epoxide hydrolase 1	Xenobiotic detoxification	Down, 0.52	0.002
NM_145499	Cyp2c70	Cytochrome P450, family 2, subfamily c, polypeptide 70	Oxidizes steroids, fatty acids and xenobiotics	Down, 0.54	0.003
BC030173	Gsta2	Glutathione S-transferase, alpha 2	Detoxification of endotoxins and xenobiotics	Down, 0.56	0.033
NM_008030	Fmo3	Flavin containing monooxygenase 3	Xenobiotic detoxification	Down, 0.57	0.005
NM_016903	Esd	Esterase D/formylglutathione hydrolase	Hydrolytic cleavage on ester bond	Down, 0.59	0.013
NM_145548	Cyp2j13	Cytochrome P450, family 2, subfamily j, polypeptide 13	Drug metabolism	Down, 0.65	0.005
NM_008184	Gstm6	Glutathione S-transferase, mu 6	Detoxification of endotoxins and xenobiotics	Down, 0.67	0.028
NM_024284	Hagh	Hydroxyacyl glutathione hydrolase	Prevent the accumulation of methylglyoxal (MG), a toxic by-product of glycolysis, amino acid and ketone body metabolism	Down, 0.68	0.015
NM_010295	Gclc	Glutamate-cysteine ligase, catalytic subunit	Free radical scavenger, rate-limiting enzyme in GSH synthesis	Down, 0.69	0.046
NM_020577	2310045H08 Rik	Methyltransferase Cyt19	Oxidative methylation of GSH bound conjugates	Down, 0.71	0.005
NM_026935	Sult1c1	Sulfotransferase family, cytosolic, 1C, member 1	Sulfate conjugation of drugs, xenobiotics, hormones and neurotransmitters	Up, 2.39	0.000
<i>Differentially expressed genes associated with Glucose metabolism</i>					
X98848	PFK-2 /FBPase-2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase	Cellular carbohydrate metabolism	Down, 0.33	0.000
BC060261	Ppp1r3b	Protein phosphatase 1, regulatory (inhibitor) subunit 3B	Carbohydrate metabolism, generation of precursor metabolites and energy	Down, 0.39	0.000
NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism	Down, 0.64	0.033
NM_009415	Tpi	Triosephosphate isomerase	Glycolysis	Down, 0.64	0.018
NM_013631	Pklr	Pyruvate kinase	Carbohydrate metabolism	Down, 0.65	0.034

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value	
NM_174870	Slc26a1	Solute carrier family 26 (sulfate transporter), member 1	Gluconeogenesis, urea synthesis	Down, 0.69	0.039	
NM_010292	Gck	Glucokinase	Carbohydrate metabolism, phosphorylation of glucose to glucose 6 phosphate	Down, 0.71	0.044	
NM_133198	Pygl	Liver glycogen phosphorylase	Starch and sucrose metabolism	Up, 1.45	0.010	
NM_020561	Smpd13a	Sphingomyelin phosphodiesterase, acid-like 3A	Carbohydrate metabolism, aminosugar metabolism	Up, 1.50	0.048	
NM_008194	Gyk	Glycerol kinase (Gyk), transcript variant 1	Glycerol uptake and metabolism, carbohydrate metabolism	Up, 3.00	6.9x10 ⁻⁴	
<i>Differentially expressed genes associated with lipid and protein metabolism</i>						
203	NM_009381	Thrsp	Thyroid hormone responsive SPOT14 homolog	<i>De novo</i> lipid synthesis	Down, 0.29	0.000
	NM_146094	Fads1	Fatty acid desaturase 1	Lipid metabolism	Down, 0.32	0.000
	NM_009338	Acat2	Acetyl-Coenzyme A acetyltransferase 2	Cellular lipid metabolism	Down, 0.52	0.000
	NM_013495	Cpt1a	Carnitine palmitoyltransferase 1a	Long chain fatty acid transportation into mitochondria	Down, 0.54	0.002
	NM_007830	Dbi	Diazepam binding inhibitor	Intracellular carrier of acyl-CoA esters	Down, 0.64	0.051
	NM_009108	Nr1h4	Nuclear receptor subfamily 1, group H, member 4	Receptor for bile acids	Down, 0.66	0.015
	NM_027827	Afmid	Arylformamidase	Lipid metabolism	Down, 0.69	0.010
	NM_007408	Adfp	Adipose differentiation related protein	Lipid transfer	Down, 0.70	0.030
	NM_028725	4632417N05 Rik	RIKEN cDNA 4632417N05 gene	Lipid metabolism, Carbohydrate transport and metabolism	Up, 1.42	0.017
	NM_021272	Fabp7	Fatty acid binding protein 7	Intracellular lipid binding and transport	Up, 1.74	0.048
	NM_023733	Crot	Carnitine O-octanoyltransferase	Beta oxidation of fatty acids	Up, 1.85	0.037
	NM_019911	Tdo2	Tryptophan 2,3-dioxygenase	Aminoacid metabolism	Up, 1.67	0.015
	NM_010401	Hal	Histidine ammonia lyase	Histidine catabolism	Up, 1.87	0.005
	NM_024434	Lap3	Leucine aminopeptidase 3	Regular turnover of intracellular proteins	Up, 1.54	0.027

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
<i>Differentially expressed genes associated with immune system</i>					
NM_011200	Ptp4a1	Protein tyrosine phosphatase 4a1	Immune function	Down, 0.49	6.9x10 ⁻⁴
NM_008972	Ptma	Prothymosin alpha	Immune function	Down, 0.64	0.052
NM_010798	Mif	Macrophage migration inhibitory factor	Immune function	Down, 0.65	0.036
NM_009883	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Transcription factor regulating genes associated with immune function	Down, 0.66	0.028
NM_010189	Fcgrt	Fc receptor, IgG, alpha chain transporter	Transport IgG	Down, 0.67	0.048
NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	Activate the expression of NF-kappa-B-responsive genes	Down, 0.67	7.5x10 ⁻⁴
NM_019688	Rapgef4	Rap guanine nucleotide exchange factor (GEF) 4	Leukocyte transendothelial migration	Down, 0.69	0.033
BC034368	Add1	Adducin 1 (alpha)	Play a role in T lymphocyte activation	Down, 0.70	0.004
NM_009735	B2m	Beta-2 microglobulin	MHC class I receptor activity, B2m is the beta-chain of MHC class I molecules	Up, 1.40	0.049
NM_008990	Pvr12	Poliovirus receptor-related 2	Immunoglobulin subtype, play essential role in coupling cell-cell adhesion	Up, 1.44	0.002
NM_009735	B2m	Beta-2 microglobulin	MHC class I receptor activity, B2m is the beta-chain of MHC class I molecules	Up, 1.40	0.049
NM_019440	AI481100	Expressed sequence AI481100	Interferon-inducible GTPase, pyrophosphatase, hydrolase activity	Up, 1.50	0.015
NM_011413	Slp	Complement component 4b	Complement system activation	Up, 1.70	0.046
NM_008768	Orm1	Orosomucoid 1	Immune system activation during acute phase reactions	Up, 1.75	0.029
NM_008198	H2-Bf	Histocompatibility 2, complement component factor B	Complement activation, humoral immune response, innate immune response	Up, 3.15	0.003
<i>Differentially expressed genes associated with growth and transport activity</i>					
NM_010517	Igfbp4	Insulin-like growth factor binding protein 4	Carrier protein for IGF-1	Down, 0.63	0.033
NM_010512	Igf1	Insulin-like growth factor 1	Growth	Down, 0.64	0.032

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
NM_012030	Slc9a3r1	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1	cAMP-mediated phosphorylation, scaffold protein	Down, 0.52	8.6x10 ⁻⁴
NM_023455	Cml4		Acetyl transferase activity	Down, 0.30	0.000
NM_022413	Trpv6	Transient receptor potential cation channel, subfamily V, member 6	Ca ²⁺ uptake in various tissues including intestine	Up, 1.47	0.015
NM_022992	Arl6ip5	ADP-ribosylation factor-like 6 interacting protein 5	Amine, amino acid, intracellular concentration regulation	Up, 1.52	0.015
AK020136		RIKEN full-length enriched library, clone:6720460K10	Mitochondrial and peroxisomal protein transport, protein folding	Up, 1.55	0.041
<i>Differentially expressed genes associated with other functions</i>					
BC060526	1700018O18 Rik	RIKEN cDNA 1700018O18 gene	Hypothetical protein	Down,0.32	0.000
NM_011066	Per2	Period homolog 2 (Drosophila)	Nucleic acid metabolism, transcription	Down,0.33	1.6x10 ⁻⁴
AK012936		RIKEN cDNA 2810049O06	Nucleic acid metabolism, transcription	Down,0.41	5.7x10 ⁻⁴
AK129036	mKIAA018	mRNA for mKIAA0018 protein	Steroid biosynthesis, lipid metabolism	Down,0.42	0.000
NM_053082	Tm4sf7	Transmembrane 4 superfamily member 7	Immune function	Down,0.44	2.1x10 ⁻⁴
NM_147015	Olf1019	Olfactory receptor 1019	Potential odorant receptor	Down,0.49	0.023
NM_026524	Mig12	Mid1 interacting protein 1	Microtubule stabilization	Down,0.53	0.003
NM_178050	Arl6ip2	ADP-ribosylation factor-like 6 interacting protein 2	Hypothetical protein	Down,0.53	5.7x10 ⁻⁴
NM_010870	Birc1e	Baculoviral IAP repeat-containing 1e	Hypothetical protein	Down,0.54	0.037
NM_007860	Dio1	Iodothyronine, type I	Converts thyroxine to tri-iodo thyronine	Down,0.55	0.003
NM_144800	Mtss1	Metastasis suppressor protein 1	Inhibits nucleation of actin filaments	Down,0.57	0.000
NM_178207	Hist1h3i	Histone 1	Nucleosome structure	Down,0.56	2.6x10 ⁻⁴
NM_133732	4931406C07 Rik	RIKEN cDNA 4931406C07 gene		Down,0.56	0.001
AK049798	Pi3k	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	Cell division and chromosome partitioning	Down,0.58	0.008
BC055045	Wasl	Wiskott-Aldrich syndrome-like	Nucleic acid metabolism	Down,0.60	0.003
NM_009930	Col3a1	Procollagen, type III, alpha 1	ECM-receptor interaction	Down,0.60	0.017

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
AK046581		RIKEN cDNA B430109A22	Hypothetical protein	Down,0.60	0.002
AK037554		Il2-inducible t-cell kinase	Leukocyte transendothelial migration	Down,0.60	0.03
AK029123	Hap 1	Tafficking protein, kinesin binding 1	Nucleic acid metabolism	Down,0.61	0.006
NM_007930	Enc1	Ectodermal-neural cortex 1	Protein metabolism	Down,0.62	0.007
AK021219	Thy	Transthyretin	Intracellular organelle	Down,0.62	0.023
BC004092	Btb	Riken cdna 1700126i16 gene	Cell cycle progression	Down,0.63	0.005
NM_178197	Hist1h2bh	Histone 1, H2bh	Nucleosome structure	Down,0.63	0.036
NM_182839	2900041A09	RIKEN cDNA 2900041A09 gene	Electron transport	Down,0.64	5.7x10 ⁻⁴
	Rik				
NM_019817	Copz1	Coatomer protein complex, subunit zeta 1	Intracellular protein transport	Down,0.64	0.002
NM_178203	Hist1h3b	Histone 1, H3b	Nucleosome formation	Down,0.64	0.006
NM_026494	6330579B17	RIKEN cDNA 6330579B17	Coenzyme A (CoA) biosynthesis from vitamin B5	Down,0.65	6.9x10 ⁻⁴
	Rik				
NM_010860	Myl6	Myosin, light polypeptide 6	Regulatory light chain of myosin	Down,0.65	0.051
NM_013556	Hprt	Hypoxanthine guanine phosphoribosyl transferase	salvage purines from degraded DNA to renewed purine synthesis	Down,0.57	8.6x10 ⁻⁵
NM_007711	Clcn3	Chloride channel 3	Signal transduction	Down,0.66	0.012
NM_009787	Pdia4	Protein disulfide isomerase associated 4	Electron transport	Down,0.67	0.027
NM_011241	Rangap1	RAN GTPase activating protein 1	mRNA processing and transport	Down,0.67	0.003
NM_178162	Hrbl	HIV-1 Rev binding protein-like	Regulation of GTPase activity	Down,0.67	0.042
BC057132	Icmt	Isoprenylcysteine carboxyl methyltransferase	Prevent endothelial cell apoptosis	Down,0.67	0.046
NM_030258	Gpr146	G protein-coupled receptor 146	Orphan receptor	Down,0.67	0.030
NM_146230	Acaa1b	Acetyl-coenzyme a acyltransferase 1b	Beta oxidation of fatty acids	Down,0.68	0.036
NM_019743	Rybp	RING1 and YY1 binding protein	Nucleic acid metabolism	Down,0.68	0.036
NM_199223	Rtn4rl2	Reticulon 4 receptor-like 2	Expressed in neurons	Down,0.69	0.001
NM_145510	Mss4	Rab interacting factor	Protein transport	Down,0.69	0.003
NM_175341	Mbnl2	Muscleblind-like 2 (Mbnl2), transcript variant 1	Play a role in myotonic dystrophy pathophysiology	Down,0.69	0.033
NM_025560	1810049H13	RIKEN cDNA 1810049H13 gene	Hypothetical protein	Down,0.69	0.031
	Rik				
NM_013588	Lrpb7	Leucine rich protein, B7 gene	Interacts with mimecan, a pituitary secreting hormone	Down,0.70	0.037

Table 6. 2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
BC004094	WD40	SEC31-like 1	Hypothetical protein	Down,0.70	0.013
NM_030087	1500032D16	RIKEN cDNA 1500032D16 gene	Hypothetical protein	Down,0.71	0.023
	Rik				
NM_029720	EGF	Cysteine-rich with egf-like domains 2	Growth factor	Down,0.71	0.048
AK032249	RPR	Riken cdna 2500001h09 gene	Regulation of nuclear pre-mRNA protein	Down,0.71	0.005
NM_029103	Armet	Arginine-rich, mutated in early stage tumors	Signal peptide	Down,0.71	0.031
AK043907	Arl6ip2	Adp-ribosylation factor-like 6 interacting protein 2	Hypothetical protein	Down,0.71	0.004
AK050289	2410127E18	Riken cdna 2410127e18 gene	Hypothetical protein	Down,0.71	0.037
	Rik				
NM_145465	Stk24	Serine/threonine kinase 24	Serine/threonine protein kinase	Down,0.71	0.001
AK004786	1200015F23	RIKEN cDNA 1200015f23 gene	Hypothetical protein	Down,0.71	0.003
	Rik				
NM_177093	C330018J07	RIKEN cDNA C330018J07 gene	Hypothetical protein	Down,0.71	0.019
	Rik				
NM_177874	F830003B07	Hypothetical protein F830003B07	Hypothetical protein	Down,0.71	0.053
NM_020578	Ehd3	EH-domain containing 3	Recycle MHC class I molecules to plasma membrane	Down,0.72	0.030
NM_177737	ZnF	Expressed sequence AI854703	Zinc ion binding	Up,1.44	0.036
NM_172379	LOC218805	Hypothetical protein LOC218805	Hypothetical protein	Up,1.48	0.023
NM_145440	Gpr14	Urotensin 2 receptor	G-protein coupled receptor activity	Up,1.48	0.042
NM_146309	Olf1337	Olfactory receptor 1337	G-protein coupled receptor activity, sensory perception of smell	Up,1.49	0.044
NM_146611	Olf1970	Olfactory receptor 970	G-protein coupled receptor activity, sensory perception of smell	Up,1.59	0.051
NM_147035	Olf1711	Olfactory receptor 711	G-protein coupled receptor activity, sensory perception of smell	Up,1.61	0.035
NM_026036	Cklfsf6	Cklf-like marvel transmembrane domain containing 6	Sensory perception, chemotaxis	Up,1.75	6.9x10 ⁻⁴
NM_025347	0610043B10	RIKEN cDNA 0610043B10 gene	Putative zinc binding protein, highly conserved in eukaryotes	Up,1.50	0.051
	Rik				
AK014425	3830408D24	Riken cdna 3830408d24 gene	Hypothetical protein	Up,1.51	0.047
	Rik				
AK035028	Fat 3	Fat tumor suppressor homolog 3	Protocadherin Fat3 isoform 2	Up,1.52	0.030

Table 6. 2. (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio (E+HS/E+TN)	p-value
NM_134046	2810429O05	RIKEN cDNA 2810429O05 gene Rik	Hypothetical protein	Up,1.54	0.035
AF510860	Flg	Profilaggrin	Cytoskeleton organization and biogenesis	Up,1.55	0.030
NM_021285	Myl1	Myosin, light polypeptide 1	Cytoskeleton organization and biogenesis	Up,1.56	0.045
NM_024445	Tsnaxip1	Translin-associated factor X (Tsnax) interacting protein 1	Calcium binding protein, EF-Hand type	Up,1.56	0.044
NM_181564	Dsg4	Desmoglein 4	Hair follicle development and maturation	Up,1.59	0.014
NM_009450	Tubb2	Tubulin, beta 2	Cytoskeleton organization and biogenesis	Up,1.97	0.013

Table 6. 3. Enriched gene ontology (GO) terms. Gene ontology terms at biological process level 5 and p value less than 0.01 are shown

Enriched GO terms (GOTERM_BP_5)	Gene number ^a	% Total ^b	p value ^c
Oxidoreductase activity	21	9.9	1.8×10^{-8}
Cholesterol and bile acid biosynthesis and steroid metabolism	17	7.9	1.6×10^{-4}
Lipid, carbohydrate and protein metabolism	38	17.7	3.0×10^{-4}
Chaperone activity	7	3.3	3.0×10^{-4}
ATP binding	18	8.5	2.6×10^{-4}
Mitochondrial proteins	20	9.4	9.3×10^{-3}
Antioxidant and detoxification activity	14	6.6	3.0×10^{-3}
Immune response	7	3.3	7.3×10^{-3}
Cell cycle regulation	6	2.8	6.5×10^{-3}
Apoptosis	6	2.8	1.2×10^{-3}
Kinases	12	5.6	4.9×10^{-3}

^aNumber of genes out of 214 in Table 6. 2 are in a given gene ontology category

^bRatio of genes involved in a gene ontology category to the total number of genes

^cP values were computed using “functional annotation chart” tool from DAVID database (<http://david.ncifcrf.gov/>)

Table 6. 4. Differentially expressed gene list in E-HS compared to E+HS rats at the end of 8 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology ^b	Ratio ^c E-HS/E+HS	p-value (<0.001)
<i>Differentially expressed genes associated with carbohydrate, and lipid metabolism</i>					
NM013631	Pklr	Pyruvate kinase liver and red blood cell	Glycolysis	Down, 0.65	<0.001
NM177960	Idi1	Fatty acid binding protein 1, liver	Steroid biosynthesis	Down, 0.79	<0.001
NM133960	Fasn	Carboxylesterase 6	Lipase activity	Down, 0.78	<0.001
<i>Differentially expressed genes associated with transcription</i>					
NM011498	Ccnd1	Basic helix-loop-helix domain containing, class b2	Negative regulation of transcription	Down, 0.77	0.001
<i>Differentially expressed genes associated with antioxidant and detoxification activities</i>					
NM009127	Scd1	Stearoyl-coenzyme a desaturase 1	Cu,Zn-SOD activity	Down, 1.61	<0.001
NM010001	Cyp2c37	Cytochrome p450, family 2, subfamily c, polypeptide 37	Detoxification	Up, 0.67	<0.001
<i>Genes associated with other functions</i>					
AK083183	930041G11R	RIKEN cDNA A930041G11 gene	Unknown function	Down, 0.55	<0.001
AK044771	930041g11	RIKEN cDNA A930041g11 gene	Unknown function	Up, 0.55	<0.001
AK077784	5830454d03	RIKEN cDNA 5830454d03 gene	Hypothetical protein	Up, 0.49	<0.001
NM010835	Msx1	Homeo box, msh-like 1	Limb pattern formation	Up, 0.56	<0.001

CHAPTER VII

CHRONIC EFFECTS OF HEAT STRESS AND/OR ERGOT ALKALOIDS ON RAT HEPATIC GENE EXPRESSION

1. ABSTRACT

Intake of ergot alkaloids associated with endophyte-infected fescue (E+) has adverse effects on animal health and productivity, which is collectively, termed fescue toxicosis. These effects are exacerbated under hot and humid (summer) conditions. A rat model for this condition was used to evaluate the effect of endophytic toxins on hepatic gene expression under long-term (three weeks) heat stress (HS). Both E+ and HS induced alterations in hepatic genes were evaluated using DNA microarrays. Rats (n = 12) were fed *ad libitum* an E+ diet and maintained under thermoneutral (TN) conditions (21°C) for five days, followed by TN or HS (31°C) conditions for three weeks. At the end of treatment period, rats were euthanized and the liver tissue was used for microarray analysis. Long-term intake of an E+ diet under TN conditions, in the present study, up-regulated genes coding for immune and electron-transport functions but down-regulated genes associated with antioxidant, xenobiotic, and chaperonic activities. Down-regulation of these essential pathways might contribute to the chronic signs associated with fescue toxicosis under TN conditions. Long-term intake of an E+ diet under HS conditions (E-

HS versus E+HS) down-regulated genes coding for immune system, as well as carbohydrate, lipid, and protein metabolism. Chronic HS (E-TN versus E-HS) in the present study down-regulated genes associated with cell proliferation, detoxification, ATP synthesis, and immune function. The E+ diet intake under long-term HS conditions up-regulated genes coding for apoptosis and detoxification but down-regulated genes associated with immune and antioxidant functions, as well as carbohydrate, lipid, and protein metabolism. Taken together the results from the present study suggested that long-term HS exposure increased the susceptibility of rats to oxidative stress. In addition, E+ diet intake further affected the animals' antioxidant and immune systems as well as chaperone activity, making them potentially more susceptible to oxidative and other stressors which contribute to fescue toxicosis.

2. INTRODUCTION

Intake of ergot alkaloids produced by the endophytic fungus *Neotyphodium coenophialum* results in a toxicological syndrome called fescue toxicosis. These ergot alkaloids, by virtue of their agonistic stimulation of alpha-1 adrenergic receptors, vasoconstrict peripheral blood vessels (Oliver, 2005). This impairs heat transfer from core to peripheral tissues, which produces hyperthermia. In addition, some ergot alkaloids are agonists for serotonin receptors that reduce vascular lumen size (through mitogenic activation of vascular smooth muscle cells) and impair thermoregulatory and satiety centers in the hypothalamus (Oliver, 2005). This also increases core body temperature and suppresses appetite during heat stress (HS). Therefore, fescue toxicosis-related

problems are exacerbated during heat stress, resulting in hyperthermia and reduced growth rate (Spiers et al., 2005a).

The liver is the most important organ for xenobiotic metabolism and detoxification, and this first pass effect of the mesenteric blood flow makes it a target organ of fescue toxicosis (Oliver, 1997). Long-term intake of an endophyte-infected (E+) diet by cattle increases liver-specific enzymes (Piper et al., 1991). Likewise, fescue toxicosis in rats actually reduces liver weight (Settivari et al., 2006). Chestnut et al. (1992) noted that this reduction in rats fed an E+ diet is similar to rats fed a control diet at the same caloric level (i.e., pair-fed group). Although it is possible that the clinical signs associated with fescue toxicosis are due to both direct and indirect (i.e., reduced caloric intake) effects on hepatic function, the objective of this preliminary study was to determine the overall impact of fescue toxicosis on hepatic gene expression under long-term heat stress conditions.

The majority of gene expression studies of HS have concentrated on the response to challenge with severe heat stress (41 - 42°C) for a short time period (30 to 60 min). However, in the real world animals are often exposed to chronic heat stress conditions (one to two months) at a lower ambient temperature between 33 to 35°C (Jordan, 2003; St-Pierre et al., 2003).

A previous study revealed that various genes associated with ATP synthesis, chaperone activity, and both antioxidant and immune systems were down-regulated in rats fed an E+ diet for five days at thermoneutrality (TN) followed by three days at HS (see chapter VI). It is expected that animals under chronic HS will exhibit different responses compared to acute HS. Spiers et al. (2005a) observed signs of adaptation in E+

rat physiological parameters under long-term TN and HS conditions. It was hypothesized that rats in the present study would exhibit similar signs of adaptation to fescue toxicosis under TN or HS conditions or to HS alone at gene level. This research is one of the first attempts to determine changes in hepatic gene expression in rats that is associated with fescue toxicosis during long-term HS.

3. MATERIALS AND METHODS

Animals and treatment diets

Male Sprague-Dawley rats (n = 12; 42 days of age at start; Charles River Laboratories, Willington, MA), were maintained on a 12 h light/dark cycle and at TN (21°C) as described earlier (Spiers et al., 2005a; Settivari et al., 2006). Feed and water were provided *ad libitum* throughout the study. Uninfected control (E-diet, containing endophyte free fescue seed) and treatment diet compositions were as previously described, with the treatment diet adjusted to provide a specific amount of ergovaline (EV; 91.5 µg/kg BW/day) (Spiers et al., 2005a). Ergovaline content in E+ seed was 4,100 ppb on a DM basis, as measured by HPLC (detection limit = 50 ppb and CV = 7%; Rottinghaus et al., 1993). All diets were stored at 4°C prior to use. Animal procedures followed guidelines for humane treatment set by the University of Missouri Animal Care and Use Committee.

Treatment period and sample collection

The experimental schedule consisted initially of an 8 day recovery period after ip implantation of telemetric temperature transmitters (Model VM-FH; Mini-Mitter

Company, Inc., Bend, OR) to record Tc and Ac. This was followed by a 5 day pretreatment period, during which Tc, FI, and BW were measured to establish a baseline for statistical analysis (Spiers et al., 2005a). During recovery and pretreatment periods, rats were fed the E- diet, whereas during the 26 day treatment period, the rats were fed E- or E+ diets. At the end of 5 days of treatment at TN, rats were divided into two groups, one maintained under continuous HS (31°C) and the other continuing at TN for 21 additional days. During treatment, Tc was collected at 10 minute intervals, with daily measurement of FI and BW at 0800 hour. At the end of the study, rats were anesthetized by im injection of a xylazine (13 mg/mL)-ketamine (87 mg/mL) cocktail (0.1 mL/100g) and exsanguinated. The liver was also collected and weighed. A section from the middle lobe of liver (~5 g) was snap-frozen in liquid nitrogen for microarray and real time-PCR experiments.

Microarray slide preparation

Microarray slides were prepared by printing 27,000 oligonucleotides (70 mer probes with an amino modification on the 5' end) specific to mouse liver (Oligator MEEBO mouse genome set, Illumina Inc., Hayward, CA) on GAPS II glass slides (Corning Incorporated, Corning, NY) using a 32-pin robotic microarray printer. Prior to hybridization, the microarray slides were re-hydrated to increase the spot size and spread the DNA more uniformly within each spot. Following the re-hydration for 30 seconds at 55°C, the slides were snap-dried on a 100°C hot plate surface for 5 seconds. Then the slides were UV cross-linked using UV cross-linker (600 mJoules). Microarray slides were then incubated in 0.2% I-block (Tropix/Serva, Heidelberg, Germany) in 1 × PBS buffer for at least one hour at 42°C to decrease background and increase specificity. The

slides were then washed with 0.2% SDS once and water twice (three minutes per wash). This was followed by drying via centrifugation and dark storage.

RNA extraction

Total RNA was extracted from the liver samples using an RNeasy Midi Kit (Qiagen Inc., Valencia, CA), purified using DNase-1 (Ambion Inc., Austin, TX) and phenol:chloroform:isoamyl alcohol (25:24:1), and concentrated using Microcon YM30 filters (Millipore Corp., Bedford, MA) as described earlier (Settivari et al., 2006). The quality and integrity of the purified RNA was checked through agarose gel electrophoresis and the quantity was measured using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE; Flanagan, 2005). The purified RNA samples were preserved at -80°C.

Microarray hybridization

A loop design was used for microarray experiments, with five biological replicates (five animals/treatment group) and three technical replicates (each RNA sample repeated on three different arrays). Concentrated and purified total RNA (15 µg) from each rat liver was reverse-transcribed to cDNA using oligo dT (IDT DNA, Coralville, IA), random hexamers (IDT DNA), 50 x amino-allyl dUTP (Sigma Chemicals, St. Louis, MO), and reverse transcription (StrataScript RT, Stratagene, La Jolla, CA). Resulting cDNA was purified using Microcon-30 filters (Millipore) and conjugated with either Cy3 or Cy5 mono reaction dyes separately by incubating in the dark for one hour. Conjugated cDNA was separated from free, unconjugated dye using a Qia-quick PCR purification kit (Qiagen) following the manufacturer's instructions. The two cDNA samples, labeled with Cy3 and Cy5 dyes, were mixed together and dried. The

dried cDNA pellet was resuspended with the hybridization mixture, which consisted of 1 × 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), poly A (10 µg/µL; Stratagene), rat Cot-I DNA (3.2 µg/µL), 20 × standard saline citrate (SSC; Stratagene), and 10% sodium dodecyl sulfate (SDS). The slides were incubated in a water bath (60°C) for 12 to 16 h. After incubation, the slides were rinsed in wash solution I (20 × SSC, and 10% SDS) and then in wash solution II (20 × SSC).

Microarray slide scanning and analysis

Microarray slides were scanned using a GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA) at 5 µm resolution, and the image was analyzed using GenePix Pro 4.0.1.12 software (Axon Instruments Inc., Union City, CA). The resulting files and the images were linked together and stored in the local BioArray Software Environment (BASE) database (Saal et al., 2002). Microarray raw data files were deposited with the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo>). The serial entry number for the microarray data was GSE8754, and the platform number was GPL5357. Each hybridization in the microarray experiment was given the following serial numbers: GSM216884, GSM216889, GSM216894, GSM217199, GSM217309, GSM217310, GSM217311, GSM217312, GSM217313, GSM217314, GSM217315, GSM217316, GSM217317, GSM217318, GSM217319, GSM217320, GSM217321, GSM217322, GSM217343, GSM217345, GSM217346, GSM217347, GSM217348, GSM217349, GSM217350, GSM217351, GSM217352, GSM217353, GSM217354, GSM217355.

Statistical analysis of microarray data

Microarray data was first filtered to remove all the blank spots. The background-corrected median intensities were normalized using pin-based LOWESS normalization within BASE. The microarray spot intensities were first log₂ transformed and genes with greater than 20% missing values were filtered using CLUSTER 3.0 software. Missing values for each gene was imputed using k-nearest neighbor method (k = 20) using significant analysis of microarrays (SAM) software. The normalized intensities were then inputted into the software package MAANOVA (Wu et al., 2003) to model the data and run statistical analyses. A two-stage ANOVA model (Wolfinger et al., 2001) was applied to the microarray data using JMAANOVA (Wu et al. 2002) software. The first stage was the normalization model to remove the effect of array and dye at the across gene level

$$y_{gntijk} = \mu + A_i + D_k + (AD)_{ik} + \varepsilon_{gntijk}.$$

The second stage was the gene-specific model in which the effects of animal-gene interaction and array-gene interaction were modeled as random effects.

$$\varepsilon_{gntijk} = (GV)_{gi} + (GD)_{gk} + r_{gntijk}.$$

For these models $y_{gntijk} = \log_2(w_{gntijk})$, w is the observed median intensity for each color channel by gene, μ is the model grand mean, A is the array, D is the dye, G is the gene, V is the animal, and ε and r are the residuals from the linear models.

A permutation F_s test (F test designed specifically for microarray data) (Cui et al., 2005) was run to test the significance of sample effect for each probe. Probes with a permutation F_s test p value less than 0.05 were regarded as significantly differentially expressed across samples.

The first stage was the use of the normalization model to remove the effects of array and dye. The residual from this model was used in the second stage analysis, which is a gene-specific model, where the effects of sample-gene interaction and array-gene interaction were modeled with both effects at random. To test the significance of sample effect for each probe (500 permutations), the F_s test (Cui et al., 2005) was used. Genes, with an adjusted permutation F_s test (False Discovery Rate; FDR) p values less than 0.05 were regarded as significantly differentially expressed across samples. This differentially expressed gene list was then filtered to remove genes with a fold change of less than 1.4 (value obtained from mean \pm 2 SD of five self-self hybridizations).

A second type of microarray analysis was performed which measures the gene expression across each individual animal (gene by animal model) using JMAANOVA program. Values obtained from this analysis were used to cluster similarly expressed genes using TIGR multi-experiment viewer software (TM4-Mev; Saeed et al., 2003). The hierarchical cluster tree obtained was confirmed using bootstrap (Kerr and Churchill, 2002) analysis. The DAVID (Dennis et al., 2003) and Entrez-Gene (<http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>) from the National Center for Biotechnology Information were used to find the gene annotation and the functions of differentially expressed genes.

Quantitative real-time PCR

Two-step qRT-PCR was used to confirm expression patterns of differentially expressed genes in the study. Expression profile of five randomly selected genes was measured with qRT-PCR. From each rat, 10 μ g of total RNA was reverse transcribed using Stratascript RT (Stratagene, La Jolla, CA) with oligo dT and random hexamer

primers. Then, 6.25 ng of cDNA was added to a 25 μ L of final PCR reaction mixture to get a final concentration of 0.25 ng/ μ L of cDNA in a SYBR green assay (Applied Biosystems, Warrington, UK). Forward and reverse primer final concentrations were 100 nM in the SYBR green assay. Primers were designed using Primer3 (Rozen and Skalesky, 2000) with an annealing temperature of 60°C and amplification size of less than 250 base pairs. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the endogenous control gene in the qRT-PCR experiments. Thermal cycling was carried out with an ABI Prism 7500 sequence detection system (Applied Biosystems Warrington, UK) under factory default conditions (50°C, 2 min; 95°C 10 min; and 40 cycles at 95°C, 15 sec; 60 °C 1 min). Each gene was measured in triplicates and the formation of single PCR product was confirmed using dissociation curves. Negative controls, which consisted of all the components of PCR mix except cDNA, were used for all the primers. The relative quantification of gene expression changes were recorded after normalizing for GAPDH gene expression computed by using the $2^{-\Delta\Delta CT}$ method (user manual #2, ABI Prism 7700 SDS). In the $2^{-\Delta\Delta CT}$ analysis, the threshold cycle (C_T) from E-TN rats was used as a calibrator sample. Statistical analyses of the data were performed by comparing E+TN, E-HS and E+HS C_T with E-TN C_T for each gene using a two-tailed t-test with unequal group variance.

4. RESULTS

Physiological responses for this study are explained in detail in Chapter 3. For better understanding, genomic responses for this study have been divided into five

sections, i.e., E-TN vs. E+TN; E-TN vs. E-HS; E-TN vs. E+HS; E+TN vs. E+HS; E-HS vs. E+HS.

Microarray analysis identified 176 genes (false discovery rate, $FDR \leq 0.15$ and fold change ≥ 1.4) as differentially expressed between E-TN and E+TN rats. Of the differentially expressed genes, 106 were up-regulated and 70 genes were down-regulated in E+TN compared to E-TN rats (Figure 7. 1). A hierarchical clustering pattern was followed to group animals and genes with similar expression pattern. Analysis identified detoxification, immune system, cell proliferation, apoptosis, chaperones (protein folding), cholesterol biosynthesis, carbohydrate and protein metabolism and electron transport pathways as affected as a result of E+ diet intake under TN conditions. The list of differentially expressed genes with their biological significance is shown in Table 7. 1.

Hepatic gene expression analysis between E-HS and E+HS rats in the present study identified 30 genes (permutation based P value ≤ 0.001 and fold change ≥ 1.4) as differentially expressed. Of these, 16 genes were up-regulated and 14 were down-regulated in E+HS compared to E-HS rats. Similarly expressed genes were clustered together (Figure 7. 2) following hierarchical clustering pattern. The differentially expressed genes represented immune system, and carbohydrate, lipid and protein metabolisms (Table 7. 2).

Comparison between E-TN and E-HS rat's revealed hepatic gene expression changes as a result of heat stress. The microarray results identified 53 genes (permutation based P value ≤ 0.001 and fold change ≥ 1.4) as differentially expressed. Of the differentially expressed genes, seven were up-regulated and 46 genes were down-regulated in E-HS compared to E-TN rats (Figure 7. 3). The genes identified as

differentially expressed specifically were associated with cell proliferation, detoxification, immune system, and electron transport (Table 7. 3).

Gene expression differences were identified in E+ rats under TN and HS conditions by comparing E+TN Vs E+HS rats. Sixteen genes (permutation based P value ≤ 0.001 and fold change ≥ 1.4) were identified as differentially expressed between the two groups. Of the differentially expressed genes, five were up-regulated and 11 were down-regulated in E+HS compared to E+TN rats (Figure 7. 4). Differentially expressed genes represented cell proliferation, immune system, electron transport, fatty acid metabolism, cytochrome P450, and chaperone functions (Table 7. 4).

This comparison represents the two extreme groups for this study (i.e., the control group with no stressor and the group that combines both the heat stress and fescue toxicosis). It is expected that this would show the largest number of differentially expressed genes. Microarray analysis for this comparison identified 160 genes (FDR $\leq 15\%$ and fold change ≥ 1.4) as differentially expressed between E-TN and E+HS rats. Among the differentially expressed genes, 53 were up-regulated and 107 genes were down-regulated in E+HS rats (Figure 7. 5). The E+ diet under HS conditions differentially expressed genes specific for cell proliferation, detoxification, immune function, electron transport, energy metabolism, and apoptosis (Table 7. 5). Real time PCR results agreed for all the five randomly selected genes with microarray results for each comparison, which validate the microarray results.

5. DISCUSSION

E-TN Vs E+TN

Long-term intake of an E+ diet at TN was hypothesized to result in changes in liver gene expression representing chronic effects of fescue toxicosis in the absence of heat stress. Microarray and real-time PCR results demonstrated that the expression of genes coding for specific physiological pathways, including detoxification, immune function, chaperone activity, steroid biosynthesis, cell proliferation, lipid and protein transport, and electron transport were altered by E+ diet at TN. These altered genomic responses could be due to direct effects of endophytic toxins or secondarily due to decreased caloric intake. These genomic effects and their relationship to the observed physiological responses are expected to provide additional insight into the pathophysiology of fescue toxicosis under TN.

Detoxification and antioxidant activity

Various genes associated with detoxification, such as alanine-glyoxylate aminotransferase, glutathione S transferase, malate dehydrogenase, and sulfide quinone reductase, were down-regulated in E+ compared to E- rats at TN. Similarly, genes associated with antioxidant activity such as stearoyl coenzyme desaturase 1 and glutathione S transferase were down-regulated, in E+ compared to E- rats at TN. Intake of an E+ diet at TN for a short period (5 day) increases the gene expression of various xenobiotic-detoxifying CYP isoforms in rat hepatic tissue (Settivari et al., 2006).

They reported that increased CYP isoforms in E+ rats might assist in metabolism of endophytic ergot alkaloids contributing to recovery observed in some of the physiological parameters, such as feed intake, by the end of short-term treatment period. However such changes in CYP gene expression did not occur during long-term treatment. One possible explanation is that rats partially adapted to endophytic toxins during the long-term treatment period. Reductions observed in feed intake, serum glucose, urea nitrogen, and alkaline phosphatase levels in E+ rats during short-term (5 day) treatment (Settivari et al., 2006) were also absent during long-term treatment (Table 3.2), suggesting adaptation in E+ rats to fescue toxicosis under TN conditions.

Genes associated with antioxidant and xenobiotic systems were down-regulated in E+ rats in the present study. Settivari et al. (2006) reported similar down-regulation of various antioxidant and xenobiotic genes in E+ rats even at the end of short-term treatment. Similarly, Oliver (1997) reported a decrease in the mean plasma antioxidant capacity in E+ steers that was seven-times below that of E- animals. Down-regulation of the genes for antioxidants in the present study could lead to the accumulation of free radicals within the cell leading to oxidative stress. The resulting oxidative stress in E+ rats is a possible mechanism for some of the clinical signs associated with fescue toxicosis. It can be speculated that oxidative stress in the long-term E+ rats to be less severe than during short-term TN study especially because in the current comparison, free radical producing CYPs were not up-regulated in E+ compared to E- rats.

Immune system

The majority of genes associated with immune function were up-regulated in E+TN compared to E-TN rats in the present study. Down-regulation of various genes connected

to pro-inflammatory responses, such as growth differentiation factor 5, interleukin 1 receptor type I; and up-regulation of dual specificity phosphatase 1 (a negative regulator of inflammation), opioid receptor, sigma 1 (an inhibitor of synthesis of pro-inflammatory cytokines) suggest reduced inflammatory reactions in E+ rats during chronic TN conditions. In support to the present findings, Filipov et al. (2000) reported anti-inflammatory effects of ergotamine in steers. They noted that pretreatment (i.v. injection; 30 min. prior to LPS injection) of steers with 40 µg ergotamine/kg BW attenuated inflammatory responses associated with LPS (0.2 µg/kg BW). They hypothesized that the observed inflammatory responses associated with acute administration of ergotamine is, at least in part, due to an ergotamine-induced cortisol increase. However in a previous study (1999), they reported that ergotamine (2 mg/kg BW) increased pro-inflammatory cytokine IL-6 in mice at the end of 10 days of treatment. Similarly, Settivari et al. (2006) observed various genes associated with pro-inflammatory response were up-regulated in E+ rats at the end of five days of treatment at TN. Decreased expression of various pro-inflammatory genes in the present study imply an adaptive mechanism in E+ rat immune function to fescue toxicosis. Similarly, up-regulation of genes associated with immune functions such as forkhead box p3, retinoid x receptor beta, immunoglobulin lambda chain, variable 1, and selectin further suggest improved immune functions in E+ rats during chronic periods.

Cell proliferation, apoptosis, and DNA repair

The long-term E+ diet intake at TN, in the present study, down-regulated various genes coding for cell proliferation, such as cyclin-dependent kinase 4, histone 1 h2bc, and gem associated protein 7. Also, genes associated with apoptosis (synovial apoptosis

inhibitor 1, programmed cell death protein 11, caspase recruitment domain family, member 10, riken cDNA C330008I15, and semaphorin 6A) were up-regulated and DNA repair mechanisms (excision repair cross-complementing rodent repair deficiency, complementation group 2, general transcription factor II H, and polypeptide 4) were down-regulated in E+ rats. Settivari et al. (2006) reported that rats fed a similar diet for 5 days at TN conditions exhibited similar gene expression pattern with down-regulation of cell proliferation genes and up-regulation of apoptotic genes. These results might at least in part contribute to reduced liver weights observed in E+ rats (Table 3. 3.).

Carbohydrate, lipid, and protein metabolism

Genes coding for carbohydrate metabolism (UDP-gal:betagal beta 1,3-galactosyltransferase polypeptide 7, fructose 1,6 biphosphate, galactose-4-epimerase, UDP), lipid metabolism (Acetyl-Coenzyme A acyltransferase 2, and hepatic lipase) and protein metabolism (dimethylglycine dehydrogenase precursor, methyl-CpG binding domain protein 3-like 1, and natriuretic peptide receptor 2) were down-regulated as a result of E+ diet intake. In support to the present findings, Settivari et al. (2006) reported down-regulation of genes coding for carbohydrate and lipid metabolism in rats fed an E+ diet for five days at TN conditions. They, however, observed up-regulation of various genes coding for gluconeogenesis and protein catabolism in E+ rats. In the present study, those genes were not differentially expressed in E+ rats. These results agree with improved feed intake in E+ rats at the end of the study (Figure 3. 2a). In the present study, genes coding for steroid biosynthesis (steroid receptor-interacting SNF2 domain and ring finger protein 4) were up-regulated in E+ rats. These results are in contrast with earlier reports (Settivari et al., 2006; Bhusari et al., 2006) which reported down-

regulation of various genes associated with steroidogenesis. However, the experimental period for which animals were exposed to E+ diet was much shorter in the above mentioned studies compared to the present study, and these results might suggest the chronic results of fescue toxicosis in rats. This could be one main reason for the recovery observed in serum cholesterol levels in E+ rats in the present study.

Electron transport functions

Genes for electron transport function, such as succinate dehydrogenase complex subunit A flavoprotein, ATPase H⁺ transporting V1 subunit G isoform 1, NADH dehydrogenase (ubiquinone) Fe-S protein 4, and rac GTPase-activating protein 1, were up-regulated in E+ rats. In contrast to the present findings, rats fed the E+ diet for 5 days exhibited down-regulation of various genes coding for electron transport function (Settivari et al., 2006). Up-regulation of electron transport genes in the present study might result in more efficient ATP synthesis. These results support the present physiological finding of improved feed conversion efficiency in the E+ rats following long-term treatment.

Other functions

Chaperonic genes (heat shock protein 70, peptidylprolyl isomerase F, endoplasmic reticulum chaperone SIL1, and heat shock protein 68) were down-regulated in E+ rats. However, these genes were expected to be up-regulated since the rats were challenged with a toxicological condition. Down-regulation of chaperonic proteins would result in accumulation of mis-folded proteins within the cell resulting in greater stress. Settivari et al. (2006) observed similar down-regulation of chaperonic proteins in E+ rats even at the end of short-term study (5 days), and the findings from the present study

indicate E+ rats did not exhibit adaptation for chaperones even during long-term fescue toxicosis exposure.

Intake of fescue toxicants in the present study increased the gene expression of serotonin receptor 5B. This could mainly be because of the agonistic interaction of fescue toxicants with serotonin receptors, and may play a major role in peripheral vasoconstriction and reduced appetite (Oliver, 2005).

The current study provided information regarding specific hepatic genes that are differentially expressed in E+ rats during chronic treatment at thermoneutrality. It has also helped to identify various pathways that are affected by long-term exposure to fescue toxicosis, as well as the adaptive mechanisms that are activated in rat liver during chronic treatment. Unlike short-term responses, E+ diet intake did not alter gene expression for cytochrome P450 isoforms and gluconeogenesis. Furthermore, gene expression for immune and electron transport functions of E+ rats was up-regulated compared to E- rats during the present study, and these results are in contrast to short-term E+ diet intake, suggesting adaptation to fescue toxicosis. In contrast, the gene expression for antioxidant, xenobiotic and chaperonic activities of E+ fed rats was lower compared to E- rats during both short- and long-term studies, contributing to the clinical signs associated with fescue toxicosis at thermoneutrality that are time independent.

E-HS vs. E+HS

Immune system

Genes associated with immune system (hepcidin antimicrobial peptide, myeloblastosis oncogene-like 1, complement component 3, and complement component factor h-like 1) were down-regulated in E+HS compared to E-HS rats. Steers that grazed

on endophyte infected fescue pastures during summer months exhibited similar decrease in immune functions (Saker et al., 1998; Allen et al., 2001). Similarly, rats fed an E+ diet (43 day) had a lowered leukocyte count (Dew et al., 1990).

Carbohydrate, fat and protein metabolism

Intake of the E+ diet under HS conditions down-regulated genes associated with carbohydrate metabolism (glucose 6 phosphate dehydrogenase and malate dehydrogenase), lipid metabolism (acyl-coenzyme A oxidase 3), and protein metabolism (methylenetetrahydrofolate dehydrogenase and serine (or cysteine) peptidase inhibitor clade a member 3k). Furthermore, down-regulation of ATP synthase (ATP synthase, H⁺ transporting, mitochondrial F₀ complex, subunit b, isoform 1) gene in E+HS compared to E-HS rats in the present study could impair the ability for efficient energy utilization. Down-regulation of these genes in the present study supports the physiological findings (i.e., significant decrease in serum glucose (Table 3. 4) and growth rate (Table 3.1) of E+ rats in the present study. In agreement with current findings, Spiers et al. (2005a) noted a decreased growth rate in E+ rats under similar heat stress conditions.

Results from the present comparison identify various genes that are affected as a result of E+ diet intake under HS conditions. The E+HS rats in the present study exhibited down-regulation of genes coding for immune system, as well as carbohydrate, lipid, and protein metabolisms even at the end of chronic exposure. Physiological parameters of E+HS (i.e., significant decrease in FI, BW and growth rate) compared to E-HS rats in the present study support the genomic findings in concluding that E+ rats did

not completely adapt to fescue toxicosis under HS conditions, even after 21 days of exposure.

E-TN vs. E-HS

Cell proliferation

Exposure to chronic HS down-regulated various genes associated with cell cycle (cyclin 11, Sry-box containing gene 5, zinc fingers and homeoboxes protein 2, eukaryotic translation initiation factor 4e, and tata box binding protein) in E-HS compared to E-TN rats. Earlier reports suggest that heat stress affects various cell cycle processes depending on the cell type, the phase of cell proliferation, and the severity of the stress (Rice et al., 1986; Kuhl et al., 2000). They observed cell cycle arrest at G1 or G2/M stages in rat glioma cells when exposed to 42°C for 2 to 48 hours. Similarly, Rowley et al. (1993) noted that heat stress blocked cell cycle and cyclin G1 expression in *Saccharomyces cerevisiae*. The above mentioned references suggests that short-term HS decrease cell proliferation and the observed reductions in the current study, suggests that E+ rats did not adapt to HS even under long-term exposures. Reduced cell proliferation could contribute to the observed reduction in liver weights in E-HS compared to E-TN rats in the present study. This reduction in liver weight occurred in spite of complete recovery in FI and FCE in E-HS rats.

Detoxification function

Cytochrome P450 2B10 gene, which plays a major role in Phase I detoxification was up-regulated as a result of HS exposure in the present study. Cytochrome P450 2B10 gene plays a major role in detoxification and bilirubin metabolism (Wagner et al., 2005).

Up-regulation of this gene would enhance detoxification capacity in E-HS rats. However, genes associated with Phase II detoxification (glutathione S transferase 3, sulfotransferase family 4a, member 1) and antiporter system (ATP-binding cassette subfamily b) was down-regulated in E-HS vs. E-TN rats. Earlier reports suggest decreased antioxidant status in heat stressed (40°C; four hours per day for 10 days) birds (Ramnath et al., 2007). Glutathione is found in almost all living cells and is considered to be a major antioxidant of the body (Ramnath et al., 2007). Glutathione in the body is chiefly associated with reducing oxidized vitamin C and E back to their reduced state, detoxifying many xenobiotics, maintaining cellular redox potential, and maintaining erythrocyte membrane integrity (Sen, 1997). Earlier reports suggest that even short-term exposure to various oxidative stressors such as ultraviolet light, viral infection, heat stress, heavy metals, surgery, inflammation, burns, and septic shock result in reduced body glutathione levels (Kosower and Kosower, 1978; Kidd, 1997; Sen, 1997).

Electron transport and ATP synthesis

Long-term heat stress down-regulated a number of genes associated with electron transport and ATP synthesis (NADH-ubiquinone oxidoreductase chain 3, NADH-ubiquinone oxidoreductase chain 4, NADH-ubiquinone oxidoreductase chain 6, NADH dehydrogenase subunit 1, NADH dehydrogenase subunit 2 kinase 2, NADH dehydrogenase subunit 5, NADH dehydrogenase subunit 4l, cytochrome c oxidase subunit i, ATPase subunit 8, cytochrome b, cytochrome c oxidase polypeptide 2, ATP synthase f0 subunit 6, and cytochrome oxidase subunit 3) in rats. Down-regulation of these genes in E-HS rats might impair their ability to utilize feed for energy. Decreased growth rates in E-HS rats in the present study, compared to E-TN rats, could be at least in

part due to the down-regulation of the above mentioned electron-transport genes.

However, this could also explain the known reduction in metabolism as often seen in heat adapted mammals to reduce excess heat load. Earlier studies suggested that heat stress (37 - 40°C) exposure is known to decrease oxidative phosphorylation efficiency, and ATP production in rats (Qian et al., 2004).

Immune function

The E-HS rats in the present comparison exhibited down-regulated genes coding for immune function (cd3 antigen, zeta polypeptide, toll-like receptor 12, T-cell receptor beta, variable 13, Riken cDNA a230106m20 gene, and immunoglobulin C2 type) compared to E-TN rats. Franci et al. (1996) compared the immune status of rabbits maintained at 33.5°C or 18.0°C for 24 days. They observed that HS decreased peripheral blood mononuclear cell proliferation and inhibited B lymphocyte differentiation. It is well known that HS, or any other major stress, stimulates adrenocortical secretion resulting in increased concentrations of serum glucocorticoids, which inhibit immune cell function (Piccolella et al., 1985; Khansari et al., 1990). Although the levels of corticosteroids were not measured in the present study, their role in immune suppression cannot be ignored. Similarly, Elvinger et al. (1992) observed a reduction in leukocyte migration to the mammary gland after chemotactic challenge in heat stressed cows during summer months.

Findings from the present comparison identified that chronic HS down-regulated genes associated with cell proliferation, detoxification, ATP synthesis, and immune function that might make these animals susceptible to oxidative stress and other stressors, while reducing energy utilization efficiency.

E+TN vs. E+HS

Combination of E+ diet and HS in the present study resulted in down-regulation of genes coding for cell proliferation (Cyclin D1, Riken cDNA 1700007i06 gene, rd rna-binding protein), oxidoreductase activity (lysyl oxidase-like 3), and fatty acid metabolism (acyl-coenzyme a oxidase 3, and pristanoyl). However, the E+HS rats exhibited up-regulation of genes coding for immune system (Ring finger protein 125), cytochrome P450 (CYP2B10) and chaperone (Zinc finger, cchc domain containing 3) systems compared to E+TN rats. It can be speculated from the above results that E+ diet intake at HS improved immune, chaperone, and detoxification systems than at TN. However, it is to be noted that all the pathways in the present comparison contain very few genes and therefore the information from this comparison cannot be used to draw conclusions regarding the affected pathways.

E-TN vs. E+HS

The present comparison would test the effects of E+ diet under HS conditions to unstressed rats maintained under TN conditions. One would hypothesize that this comparison would represent the two extreme conditions.

Immune functions

Intake of the E+ diet intake under HS conditions down-regulated various genes associated with immune function (immunoglobulin lambda chain, variable 1, haptoglobin, prostaglandin e synthase, regenerating islet-derived family, member 4, and catenin delta 1). Furthermore, intake of the E+ diet up-regulated various pro-

inflammatory genes (cd14 antigen, interleukin 2 receptor beta chain) in E+HS rats compared to E-TNrats. It is interesting to note that immune function was up-regulated in E+ compared to E- rats under TN conditions (see E-TN vs. E+TN), but was down-regulated under HS conditions (see E-HS vs. E+HS). Taken together, these results suggest that HS contributed to the suppressed immune response and increased inflammation in E+HS rats. Altered immune responses and inflammatory changes are commonly observed in fescue toxicosis (Filipov et al., 1999). Rodents fed E+ toxins exhibited a lowered red and white blood cell counts, reduced response to mitogens, and increased T suppressor cell numbers in the spleen compared to E- rats (Dew et al., 1999). Oliver (2005) reported that many of the clinical signs associated with fescue toxicosis, such as hyperalgesia, lameness, and coagulation defects (due to blood stasis in peripheral vessels) are due to a decrease in immune activity and an increase in inflammation functions. Results of the present experiment support Oliver's (2005) reports at the gene level.

Antioxidant and detoxification systems

Various genes associated with antioxidant system, such as stearoyl-coenzyme a desaturase 1, microsomal glutathione s-transferase 3, catalytic subunit of glutamate-cysteine ligase, protein-tyrosine sulfotransferase 2, selenoprotein p1, haptoglobin, peroxiredoxin 6, sulfide quinone reductase, and amiloride binding protein 1, were down-regulated in E+HS rats compared to E-TN rats. Down-regulation of these genes would impair the animal's ability to neutralize free radicals produced as a result of heat stress and E+ toxicants resulting in oxidative stress. Similarly, various genes associated with detoxification functions (glutaminase 2, glutathione s-transferase mu 3, glutathione s-

transferase kappa 1, glutathione s-transferase, theta 2, alanine-glyoxylate aminotransferase polypeptide 1, and cytochrome p450 2g1) were down-regulated in E+HS compared to E-TN rats. However, various isoforms of UDP-glucuronosyl transferase, (UDP-glucuronosyltransferase 1a9, UDP- glycosyltransferase 1 a10, UDP-glucuronosyltransferase 1a6a, UDP glucuronosyltransferase 1a12, and UDP glucuronosyltransferase 1a5) were up-regulated in E+HS compared to E-TN rats. Up-regulation of UDP detoxification system along with cytochrome P450 2d6 and sulfotransferase 4a1 genes in E+HS compared to E-TN rats could be an adaptive mechanism under long-term exposures to alleviate clinical signs associated with fescue toxicosis. Settivari et al. (2006) observed decreased antioxidant gene expression in E+ rats even at TN. Lakritz et al. (2002) observed decreased blood levels of glutathione in E+ cattle under HS conditions. Similarly, Oliver (1997) noted decreased mean plasma antioxidant capacity in E+ steers that was seven times below that of E- animals. In contrast, up-regulation of UDP glucuronosyl transferase enzymes in the present study could be an adaptation mechanism in E+ rats to fescue toxicosis under HS conditions.

Apoptosis and cell proliferation

Genes associated with cell proliferation (cyclin D1, cyclin A2, basic leucine zipper and w2 domains 2, tumor protein p53 inducible nuclear protein 2, tax1 binding protein 1, cyclin-dependent kinase 4, and netrin g1) were down-regulated in E+HS compared to E-TN rats. Various genes coding for programmed cell death such as ubiquitin c, perp tp53 apoptosis effector, and cd164 antigen were down-regulated in E+HS vs. E-TN rats. Similarly, genes coding for negative regulation of apoptosis (neuronal apoptosis inhibitory protein related sequence 4, synovial apoptosis inhibitor 1),

chaperone activity (interleukin 2 receptor beta chain), and DNA repair and cell survival (menage a trois 1, rev3-like, catalytic subunit of DNA polymerase zeta rad54 like, general transcription factor ii h polypeptide 4, nucleoside diphosphate linked moiety x-type motif 12) were up-regulated in E+HS compared to E-TN rats. The E+ diet intake under TN conditions reduced cell proliferation and increased apoptosis, and therefore the effect on cell proliferation and apoptosis during HS was expected to be greater than at TN. However in the present comparison, decreased apoptotic rates were observed in E+HS rats possibly due to up-regulation of DNA repair mechanisms and chaperone activity during the present chronic exposures.

Carbohydrate, lipid and protein metabolism

The E+HS rats in the present study exhibited down-regulation of several genes associated with carbohydrate metabolism (transmembrane protein 123, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2, solute carrier family 37 member 4, and afamin precursor), ATP synthesis (ATP synthase and NADH dehydrogenase subunit 2kinase 2) and growth (insulin-like growth factor binding protein 4 and latent transforming growth factor beta binding protein 1) compared to E-TN rats. Similarly, genes coding for lipid metabolism (thyroid hormone responsive spot14 homolog, fatty acid binding protein 1, fatty acid synthase, acetyl-coenzyme a acyltransferase 2, acyl-coenzyme a oxidase 3, pristanoylprotein 1, fatty acid desaturase 1, prostaglandin e synthase, lipase, oxysterol binding protein-like 11, carnitine palmitoyltransferase 1a, sulfotransferase family 4a, member 1) and cholesterol biosynthesis (sterol regulatory element binding factor 1, cytochrome p450, family 51) were down-regulated in E+HS compared to E-TN rats. Similarly, various genes coding for protein metabolism (serine/cysteine peptidase

inhibitor A3k, bacterial acetolactate synthase-like, alanyl-trna synthetase, cathepsin g, fumarylacetoacetate hydrolase, tolloid-like 2, casein kinase ii, beta subunit, zinc finger, dhhc domain containing 3, axin 1, sry-box containing gene 5, eukaryotic translation elongation factor 2, and heterogeneous nuclear ribonucleoprotein d) were down-regulated in E+HS compared to E-TN rats. Reduced growth and production efficiencies, characteristic of fescue toxicosis could be at least in part due to the decreased expression of carbohydrate, lipid and protein metabolism, as well as ATP synthesizing genes in E+HS rats. Down-regulation of genes associated with cholesterol biosynthesis is in agreement with reduced serum cholesterol levels in E+HS compared to E-TN rats in the present study.

Taken together, these results suggest that E+ rats under HS conditions, exhibited signs of adaptation to apoptosis and detoxification. However, immune and antioxidant systems were still down-regulated in this group, making the animals susceptible to oxidative stress and other stressors. Similarly, depressed carbohydrate, lipid and protein metabolisms make the rats inefficient to utilize their feed energy contributing to reduced production efficiency.

6. CONCLUSIONS

The present study identified genomic pathways that are affected due to heat stress and/or fescue toxicosis and possible adaptation mechanisms to these stressors in rat hepatic tissue. Chronic HS in the present study down-regulated various genes coding for cell proliferation, detoxification, immune function, electron transport and ATP synthesis.

The E+ diet intake during the present chronic study, down-regulated genes associated with antioxidants, cell proliferation, carbohydrate, lipid, and protein metabolisms under both TN and HS conditions. Under TN conditions, E+ diet intake down-regulated genes associated with detoxification, DNA repair mechanism, and chaperones however under HS conditions, these pathways were up-regulated. Possible role of decreased caloric intake in the present findings could not be separated and therefore future studies are recommended using pair-fed groups.

Figure 7. 1. Differentially expressed genes between E-TN and E+TN rats (FDR \leq 15% and fold change greater than 1.4%) clustered using TIGR multi-experiment viewer. Boot strap analysis was done with 1000 iterations to assess the significance of hierarchical tree. The clustering pattern divided the rats into two groups with 100% support based on their treatment (top cluster). Of the differentially expressed genes, 106 were up-regulated while 70 were down-regulated by chronic heat stress. Green blocks represent the down-regulated genes and the red blocks represent up-regulated genes and the black boxes represent genes whose expression did not change due to chronic heat stress exposure. Symbol E- indicate rats fed non endophyte infected fescue diet and E+ indicate rats fed an endophyte infected fescue diet under thermoneutral (TN) conditions.

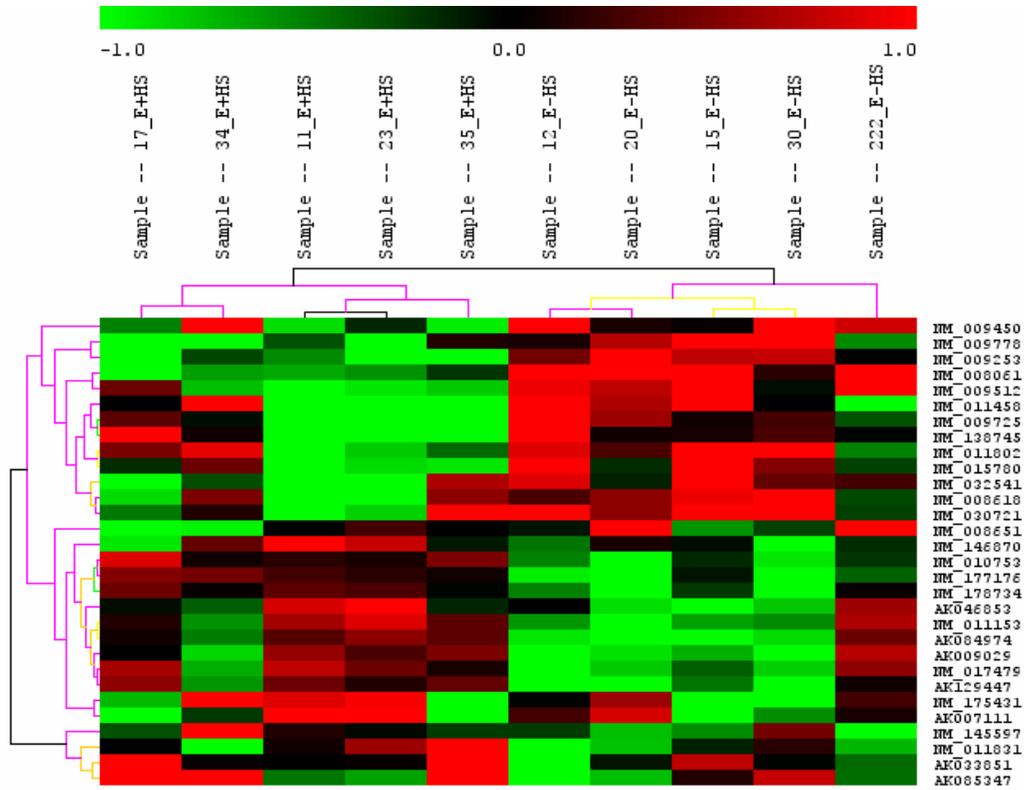


Figure 7. 2. Differentially expressed genes (p value ≤ 0.001 and fold change greater than 1.4%) between E-HS and E+HS rats. Boot strap analysis was done with 1000 iterations to assess the significance of hierarchical tree. The clustering pattern divided the rats into two groups with 100% support based on their treatment (top cluster). Of the differentially expressed genes, 16 were up-regulated while 14 were down-regulated by chronic heat stress. Green blocks represent the down-regulated genes and the red blocks represent up-regulated genes and the black boxes represent genes whose expression did not change due to chronic heat stress exposure. Symbol E- indicate rats fed non endophyte infected fescue diet and E+ indicate rats fed an endophyte infected fescue diet under heat stress (HS) conditions.

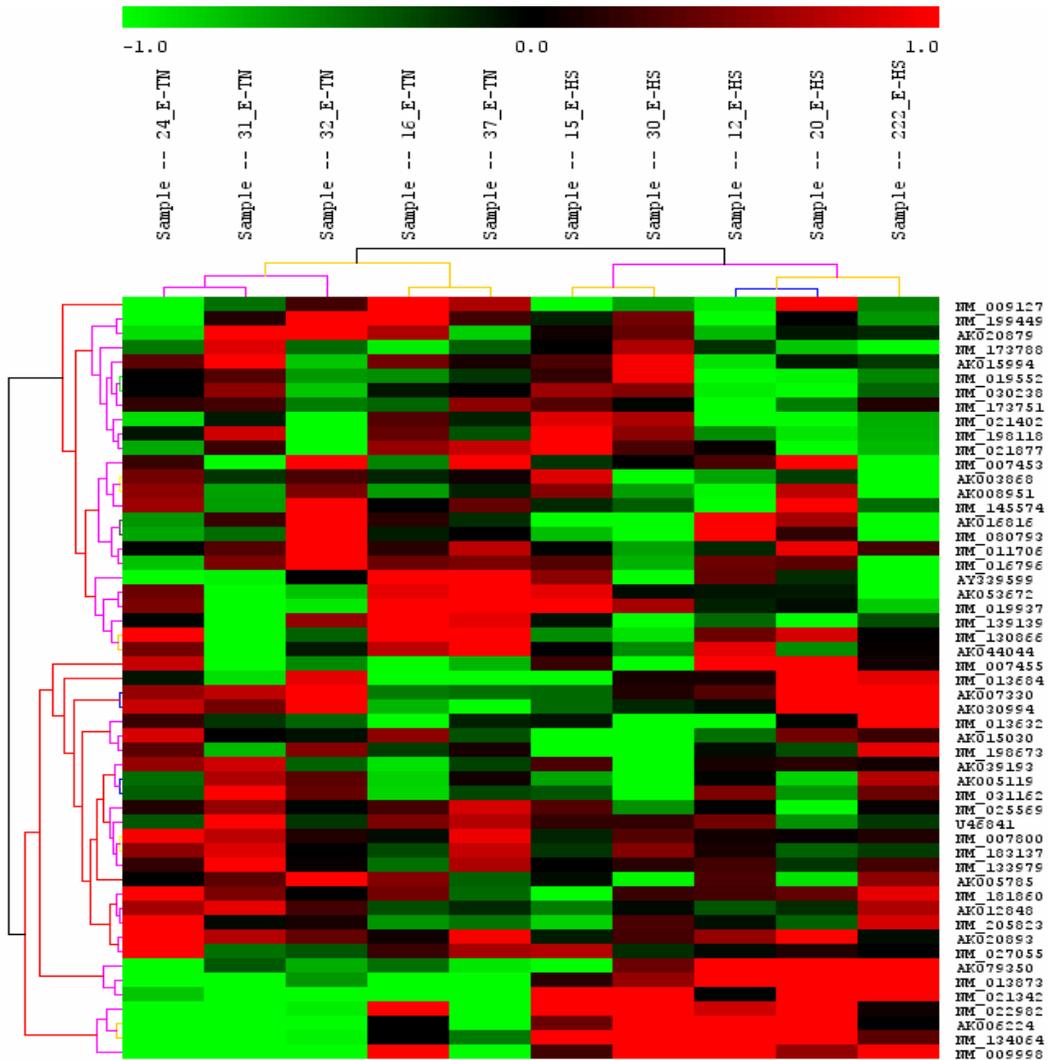


Figure 7.3. Differentially expressed genes (p value ≤ 0.001 and fold change greater than 1.4%) between E-TN and E-HS rats. Boot strap analysis was done with 1000 iterations to assess the significance of hierarchical tree. The clustering pattern divided the rats into two groups with 100% support based on their treatment (top cluster). Of the differentially expressed genes, 7 were up-regulated while 46 were down-regulated by chronic heat stress. Green blocks represent the down-regulated genes and the red blocks represent up-regulated genes and the black boxes represent genes whose expression did not change due to chronic heat stress exposure. Symbol E- indicate rats fed non endophyte infected fescue diet under thermoneutral (TN) or heat stress (HS) conditions.

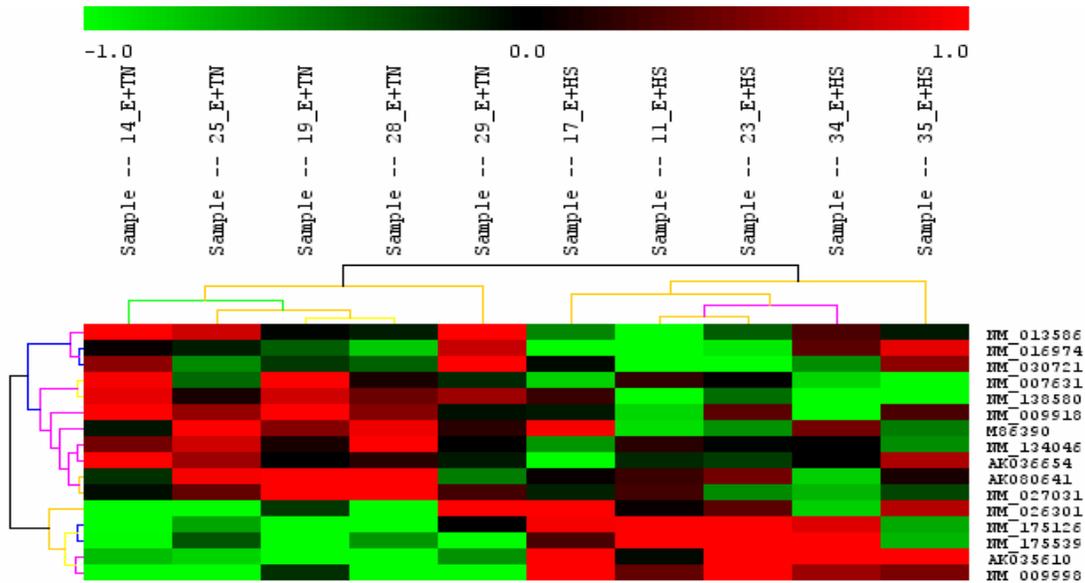


Figure 7. 4. Differentially expressed genes between E+TN and E+HS rats (p value ≤ 0.001 and fold change greater than 1.4%) clustered using TIGR multi-experiment viewer. Boot strap analysis was done with 1000 iterations to assess the significance of hierarchical tree. The clustering pattern divided the rats into two groups with 100% support based on their treatment (top cluster). Of the differentially expressed genes, 5 were up-regulated while 11 were down-regulated by chronic heat stress. Green blocks represent the down-regulated genes and the red blocks represent up-regulated genes and the black boxes represent genes whose expression did not change due to chronic heat stress exposure. Symbol E+ indicate rats fed an endophyte infected fescue diet under thermoneutral (TN) or heat stress (HS) conditions.

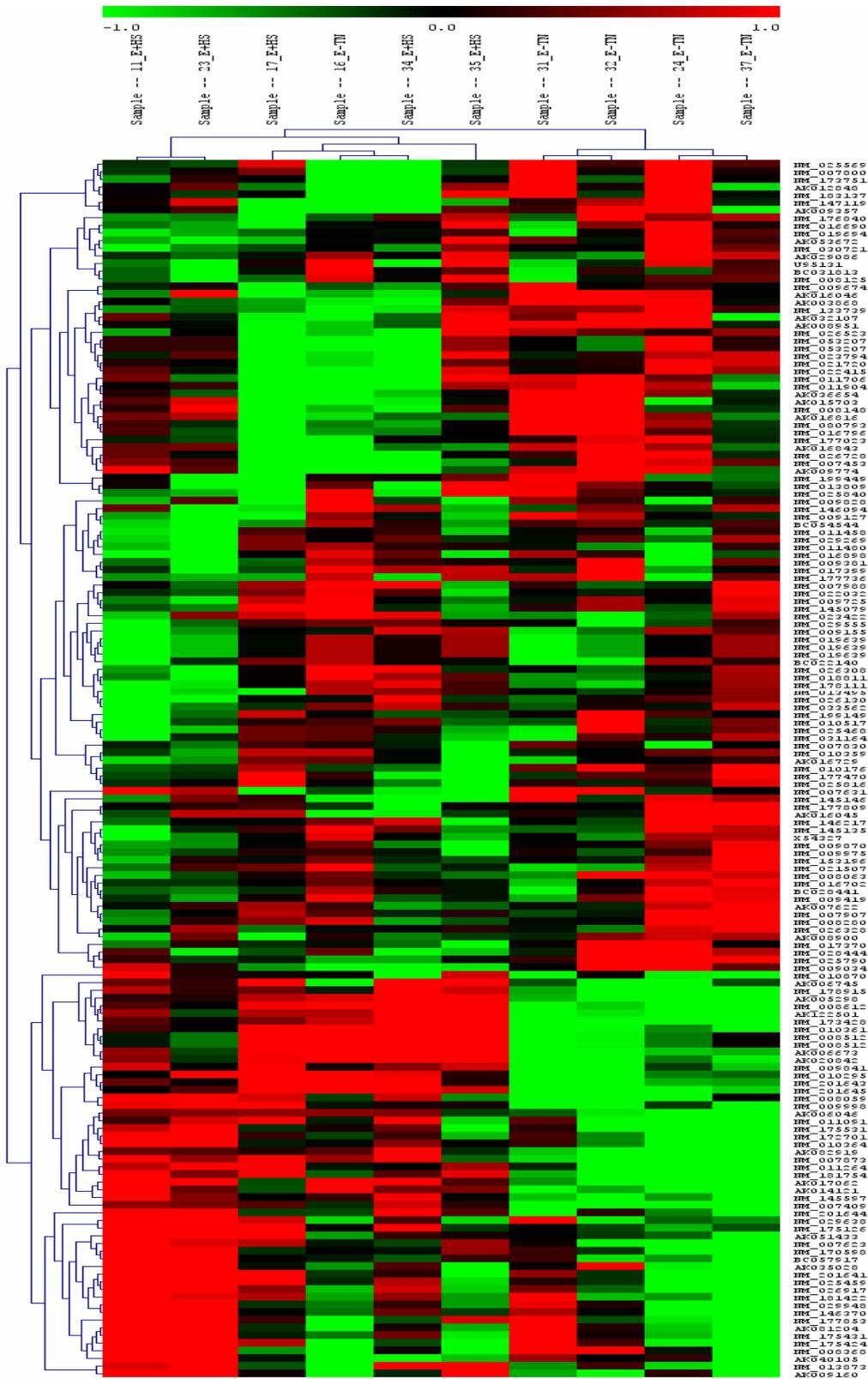


Figure 7. 5. Differentially expressed genes (FDR < 15% and fold change greater than 1.4%) between E-TN and E+HS rats clustered using TIGR multi-experiment viewer. Boot strap analysis was done with 1000 iterations to assess the significance of hierarchical tree. The clustering pattern divided the rats into two groups with 100% support based on their treatment (top cluster). Of the differentially expressed genes, 53 were up-regulated while 107 were down-regulated by chronic heat stress. Green blocks represent the down-regulated genes and the red blocks represent up-regulated genes and the black boxes represent genes whose expression did not change due to chronic heat stress exposure. Symbol E- indicate rats fed non endophyte infected fescue diet and E+ indicate rats fed an endophyte infected fescue diet under thermoneutral (TN) or heat stress (HS) conditions.

Table 7. 1. Differentially expressed gene list in E-TN compared to E+TN rats at the end of 26 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
<i>Differentially expressed genes associated with detoxification and antioxidant activity</i>					
NM_016702	Agxt	Alanine-glyoxylate aminotransferase	Gluconeogenesis (in the mitochondria) and glyoxylate detoxification (in the peroxisomes)	Down, 0.61	0.005
NM_008618	Mdh1	Malate dehydrogenase	Detoxification	Down, 0.41	0.103
NM_029555	Gstk1	Glutathione S-transferase kappa 1	Glutathione transferase activity, peroxidase activity	Down, 0.66	0.009
NM_021507	Sqrd1	Sulfide quinone reductase-like	Generation of precursor metabolites and energy	Down, 0.66	0.018
NM_009127	Scd 1	Stearoyl - Coenzyme A desaturase 1	Copper, zinc superoxide dismutase activity	Down, 1.83	0.033
BC036148	Osr1	Oxidative-stress responsive 1	Oxidoreductase activity	Up, 0.63	0.057
<i>Differentially expressed genes associated with immune system</i>					
NM_008109	Gdf5	Growth differentiation factor 5	Immune through B cell lineage	Down, 0.80	0.071
NM_008362	Il1r1	Interleukin 1 receptor, type I	Receptor for IL-1A, IL-1B, and IL-1 receptor antagonist (IL-1RA), activation of NF-kappa-B	Down, 0.46	0.066
NM_144800	Oprs1	Opioid receptor, sigma 1	Upregulation of IL-10	Up, 0.50	0.048
NM_011347	Selp	Selectin, platelet	Mediates the interaction of platelets with leukocytes, monocyte infiltration	Up, 0.67	0.064
NM_054039	Fox p3	Forkhead box p3	Critical regulator of CD4+CD25+ regulatory T cell development and function	Up, 0.43	0.066
NM_011306	Rxb	Retinoid X receptor beta	Binds to class I regulatory element of MHC class I genes and the estrogen response element	Up, 0.47	0.085
NM_026301	Rnf125	Ring finger protein 125	Ubiquitination	Up, 2.1	0.001

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_013642	Dusp1	Dual specificity phosphatase 1	Negative regulation of inflammation	Up, 0.87	0.062
AK007622	Igl-V1	Immunoglobulin lambda chain, variable 1	Beta chain of MHC	Up, 0.76	0.041
<i>Differentially expressed genes associated with blood</i>					
NM_007657	Cd9	CD9 antigen	Heme metabolism	Down, 0.73	0.07
NM_013551	Hmbs	Hydroxymethylbilane synthase	Heme biosynthesis	Down, 0.41	0.058
NM_011581	Thbs2	Thrombospondin 2	Required for normal platelet formation and function	Up, 0.56	0.08
<i>Differentially expressed genes associated with cell proliferation</i>					
NM_009870	Cdk4	Cyclin-dependent kinase 4	Cell cycle, Forms a stable complex with D-type G1 cyclins	Down, 0.49	0.051
NM_023422	Hist1h2bc	Histone 1 h2bc	Chromosome organization and biogenesis	Down, 0.43	0.055
NM_007949	Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	Nucleotide excision repair of DNA	Down, 0.72	0.033
NM_010364	Gtf2h4	General transcription factor II H, polypeptide 4	Nucleotide excision repair (NER) of DNA	Down, 0.45	0.066
NM_027189	Gemin7	Gem associated protein 7	Spliceosomal assembly	Down, 0.60	0.033
NM_027422	2700087H15Rik	Riken cDNA 2700087H15 gene	DNA binding nuclear protein	Up, 0.45	0.033
NM_008612	Mnat1	Menage a trois 1	Transcriptional activity	Up, 0.41	0.079
AK005562	Eml	Microtubule associated protein EMAP	Cell proliferation	Up, 40	0.048
AK010853	2500002G23Rik	Zinc finger protein 4	Transcriptional activity	Up, 0.48	0.048
<i>Differentially expressed genes associated with chaperone activity</i>					
NM_008301	Hspa2	Heat shock protein 70	Folding of newly translated polypeptides	Down, 0.73	0.067

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_134084	Ppif	Peptidylprolyl isomerase F (cyclophilin F)	Accelerate the folding of proteins	Down, 0.45	0.087
NM_030749	Sil1	Endoplasmic reticulum chaperone SIL1	Molecular chaperone	Down, 0.74	0.043
M12573	Hsp68	Heat shock protein 68	Involved in unfolded protein response	Down, 0.52	0.004
<i>Differentially expressed genes associated with lipid, cholesterol and steroid metabolism</i>					
NM_177470	Acaa2	Acetyl-Coenzyme A acyltransferase 2	Fatty acid elongation, metabolism, bile acid biosynthesis	Up, 0.53	0.057
NM_008280	Lipc	Lipase, hepatic	Important enzyme in HDL metabolism	Up, 0.49	0.062
AK078028	Rnf4	Ring finger protein 4	Enhances steroid receptor-mediated transcriptional activation.	Up, 0.52	0.066
NM_030730	Srisnf2l	Steroid receptor-interacting SNF2 domain	Steroid biosynthesis	Up, 0.55	0.072
<i>Differentially expressed genes associated with transporter proteins</i>					
AK033301	Klc2	Kinesin light chain 2	Organelle transport	Down, 0.41	0.083
NM_009998		Vacuolar protein sorting 4b	Intracellular protein transport	Down, 0.77	0.099
NM_028772	Dmgdh	Dimethylglycine dehydrogenase precursor	Cellular localization, intracellular protein transport	Down, 0.96	0.048
NM_007618	Serpina6	inhibitor	Major transporter for glucocorticoids	Down, 0.49	0.085
NM_007455	Ap1g2	Adaptor protein complex AP-1, gamma 2 subunit	Protein sorting in the late-Golgi/trans-Golgi network	Down, 0.52	0.002
NM_145491	Rhoq	Ras homolog gene family, member Q	Insulin stimulated glucose transport	Up, 0.50	0.082
NM_008512	Lrp1	Low density lipoprotein receptor-related protein 1	Endocytosis and recycling of Platelet-derived growth factor-BB	Up, 0.41	0.085
NM_025286	Slc31a2	Solute carrier family 31, member 2	Low-affinity copper uptake	Up, 0.44	0.071
NM_053195	Slc24a3	Solute carrier family 24 (sodium/potassium/calcium exchanger),	Ion transport	Up, 0.46	0.057
NM_007461	Apba2	Amyloid beta (A4) precursor protein-binding, family A, member 2	Carrier protein	Up, 0.54	0.085
NM_172890	Gabt4	Gamma-aminobutyric acid (GABA-A) transporter 4	GABA transporter protein	Up, 0.52	0.071

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
AF249747	KCNQ4	Potassium voltage-gated channel, subfamily q, member 4	Transporter function	Up, 0.46	0.052
NM_015748	Slit1	Slit homolog 1	Ion transport	Up, 0.67	0.057
<i>Differentially expressed genes associated with carbohydrate and protein metabolism</i>					
NM_133748	Insig2	Insulin induced gene 2	Essential elements of the feedback inhibition system of cholesterol synthesis	Down, 0.44	0.084
NM_028772	Dmgdh	Dimethylglycine dehydrogenase precursor	Amine metabolism, amino acid and derivative metabolism	Down, 0.50	0.055
NM_028557	Mbd311	Methyl-CpG binding domain protein 3-like 1	Negative regulation of cellular physiological process	Up, 0.43	0.071
NM_053092	Kars	Lysyl-tRNA synthetase	Amine metabolism	Up, 0.44	0.066
BC056137	6493428	Expressed sequence AI413414	Nucleic acid metabolism	Up, 0.54	0.051
NM_178389	Gale	Galactose-4-epimerase, UDP	Nucleotide sugar metabolism	Up, 0.43	0.057
NM_170598	Rbm12	RNA binding motif protein 12	Nuclear protein, phosphorylation, rna-binding	Up, 0.41	0.078
NM_008084	Fbp	Fructose 1,6-biphosphate	Glycolysis	Up, 0.85	0.057
NM_134084	Ppif	Peptidylprolyl isomerase F	Aminosugar metabolism	Down, 0.45	0.087
NM_146184	B3galt7	UDP-gal:betagal beta 1,3-galactosyltransferase polypeptide 7	Carbohydrate metabolism	Up, 0.75	0.062
NM_173788	Npr2	Natriuretic peptide receptor 2	Protein metabolism	Down, 0.73	0.066
<i>Differentially expressed genes associated with ubiquitination and apoptosis</i>					
NM_026301	Rnf125	Ring finger protein 125	ubiquitination and proteasomal degradation of target proteins	Down, 0.52	0.040
NM_022032	Perp	PERP, TP53 apoptosis effector	p63 developmental program, epithelial integrity	Down, 0.52	0.051
BC057917	Syvn1	Synovial apoptosis inhibitor 1	Apoptosis inhibitor	Down, 0.70	0.030
NM_010822	Mpg	N-Methylpurine-DNA glycosylase	Cell death	Down, 0.45	0.070
NM_207215	Phr1	Pam, highwire, rpm 1	E3 ubiquitin ligase protein	Up,0.79	0.042
NM_011053	Pdcd11	Programmed cell death protein 11	Apoptosis	Up,0.42	0.055
NM_130859	Card10	Caspase recruitment domain family, member 10	Apoptosis	Up,0.45	0.034

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_011053	Pdcd11	Programmed cell death protein 11	Apoptosis	Up, 1.42	0.017
NM_144917	C330008I1 5Rik	Riken cDNA C330008I15	Apoptosis	Up, 1.74	0.048
NM_028557	Mbd3l1	Methyl-CpG binding domain protein 3-like 1	Transcriptional repressor, negative regulation of metabolism	Up, 0.43	0.072
NM_018744	Sema6a	transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	Apoptosis	Up, 1.85	0.037
<i>Differentially expressed genes associated with electron transport</i>					
BC031849	MGC:3035	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Coenzyme metabolism, cofactor catabolism, electron transport	Up, 0.45	0.073
NM_024173	ATP6v1g1	ATPase, H ⁺ transporting, V1 subunit G isoform 1	Catalytic subunit of the peripheral V1 complex of vacuolar ATPase	Up,0.55	0.066
AK020108	Ndufs4	NADH dehydrogenase (ubiquinone) Fe-S protein 4	Electron transport	Up, 1.67	0.015
NM_053164	Mrp143	Mitochondrial ribosomal protein l43	Mitochondrial protein synthesis	Up, 0.40	0.066
NM_012025	Racgap1	Rac GTPase-activating protein 1	Electron transport, generation of precursor metabolites and energy	Up,0.43	0.080
<i>Differentially expressed genes associated with other functions</i>					
NM_010929	Notch4	Notch gene homolog 4	Hypothetical protein	Up,0.45	0.048
AK083573	D030046P 11Rik	Fibulin 1	Hypothetical protein	Down,0.75	0.110
NM_025623	Nipsnap3b	Nipsnap homolog 3B	Unknown function	Down,0.42	0.100
NM_011535	Tbx3	t-box 3	Embryo development	Up,0.45	0.070
AK012465	Tm4sf7	Pterin 4 alpha carbinolamine dehydratase	Hepatocyte nuclear factor transcription	Up,0.43	0.085
NM_028868	Cxxc1	CXXC finger 1	Transcriptional activity	Up,0.54	0.087
BC054544	Ctnnd1	Catenin delta 1	Tyrosine kinase activity	Down,0.62	0.035

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_009918	Cnga3	Cyclic nucleotide gated channel alpha 3 interacting protein 2	Visual signal transduction	Up,0.43	0.056
NM_199449	Zhx2	zinc fingers and homeoboxes protein 2	Transcriptional activity	Down,0.57	0.078
AK053672	E130119P06Rik	Hypothetical G-protein	Hypothetical protein	Down,0.47	0.010
AK016816	4933415D12Rik	Sry-box containing gene 5	Unknown function	Down,0.59	0.076
NM_007623	Cbx2	Chromobox homolog 2	Transcriptional activity	Up,0.60	0.033
X99104	Gli2	Gli-kruppel family member gli2	Embyo development	Up,0.46	0.041
NM_026665	3110002L15Rik	Riken cDNA 4921510p06	Cell division and chromosome partitioning	Up,0.43	0.087
NM_025468	SPC21	Sec11-like 3	Transcriptional activity	Down,0.47	0.056
NM_023697	Rdh14	Retinol dehydrogenase 14	Hypothetical protein	Down,0.63	0.004
AK080855		Myosin phosphatase targeting/regulatory subunit	Hypothetical protein	Up,0.50	0.067
AK010761	2410104C19Rik	ES cells cDNA	Unknown function	Down,0.46	0.066
NM_144790	A930021G21Rik	Ankyrin repeat domain 33	Unknown function	Down,0.46	0.060
NM_030238	Dync1h1	Dynein cytoplasmic 1 heavy chain 1	Acts as a motor for the intracellular retrograde motility of vesicles and organelles	Down,0.85	0.057
AK047140	Mark1	MAP/microtubule affinity-regulating kinase 1	Cytoskeleton stability	Up,0.44	0.089
NM_052976	Ophn1	Oligophrenin 1	Stimulates GTP hydrolysis of members of the Rho family	Up,0.53	0.007
M86390	Msn	Moesin mRNA, 3' end	Unknown function	Up,0.90	0.057
AK006941	1700072N11 Rik	Ribosomal protein s2	Unknown function	Up,0.43	0.049
NM_009797	Copz1	Capping protein	Intracellular protein transport	Down,0.42	0.090
NM_008451	Klc2	Kinesin light chain 2	Organelle transport	Down,0.41	0.083
BC035304	Ttc9c	Tetratricopeptide repeat domain 9c	Hypothetical protein	Down,0.67	0.055
AK033094	Helz	Helicase with zinc finger domain	Regulatory light chain of myosin	Down,0.42	0.094

Table 7.1 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_026746	1110014D 18Rik	RIKEN cDNA 1110014d18 gene	Hypothetical protein	Up,0.47	0.062
AK032600	6430630M 04Rik	Cytidine and dcmp deaminase domain containing 1	Unknown function	Down,0.42	0.070
NM_194346	Rnf31	ring finger protein 31	Hypothetical function	Down,0.59	0.066
AK129075	KIAA0173	mRNA for mKIAA0173 protein	Unknown function	Up,0.44	0.057
BC019368	2410072D 24Rik	Riken cDNA 2410072D24	Hypothetical protein	Down,0.61	0.062
AK005635	Tmprss13	Transmembrane protease, serine 13	Protein transport	Down,0.42	0.030
NM_022416	Stk32b	Serine/threonine kinase 32B	Kinase activity	Down,0.53	0.105
NM_130866	Olf78	Olfactory receptor 78	Olfactory function	Down,0.44	0.082
NM_010483	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	Neurotransmitter	Up,0.43	0.105
NM_146870	Olf25	Olfactory receptor 25	Olfactory function	Down,0.44	0.075
NM_008124	Gjb1	Gap junction membrane channel protein beta 1	Transmembrane channels	Down,0.44	0.087
BC026767	4122402O 22 Rik	Riken cDNA 4122402O22 gene	Hypothetical protein	Up,0.93	0.066
NM_007874	Reep5	Receptor accessory protein 5	Promote functional cell surface expression of olfactory receptors	Down,0.40	0.107
NM_146410	Olf1420	Olfactory receptor 1420	Olfactory function	Up,0.54	0.060
NM_133739	2310075C 12Rik	Riken cDNA 2310075C12 gene	Hypothetical protein	Down,0.47	0.062
AK044565	Bri3bp	Bri3 binding protein	Unknown function	Up,0.43	0.062
NM_011113	Plaur	Urokinase plasminogen activator receptor	Plasmin formation	Up,0.43	0.058
NM_175520	Gpr81	G protein-coupled receptor 81	Signal peptide	Up,0.49	0.070
NM_011014	Oprs1	Opioid receptor, sigma 1	Analgesic activity	Down,0.41	0.048
NM_009450	Tubb2	Tubulin, beta 2	Cytoskeleton organization and biogenesis	Down,0.57	0.056
AK039193	A230106M 0RRik	Riken cDNA a230106m20 gene	Unknown function	Down,0.55	0.086
NM_013551	Hmbs	Hydroxymethylbilane synthase	Heme biosynthesis	Down, 0.41	0.058
NM_145543	Clcc1	Mid-1-related chloride channel 1	Unknown function	Up, 0.41	0.081

Table 7. 2. Differentially expressed gene list in E+HS compared to E-HS rats during long-term exposure

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
<i>Differentially expressed genes associated with Immune system</i>					
NM_032541	Hamp	Hepcidin antimicrobial peptide	Antimicrobial activity, maintenance of iron homeostasis, regulation of both intestinal iron absorption and storage in macrophages	Down, 0.55	0.000
NM_008651	Mybl1	Myeloblastosis oncogene-like 1	Proliferation and/or differentiation of B-lymphoid cells.	Down, 0.89	0.001
NM_009778	Cox6c	Complement component 3	B-cell stimulatory activity, a mediator of local inflammatory process, causes histamine release from mast cells and basophilic leukocytes, regulation of endocytosis.	Down, 0.51	0.000
NM_015780	Cfh11	Complement component factor h-like 1	Prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells	Down, 0.41	0.000
<i>Differentially expressed genes associated with carbohydrate, fat and protein metabolism</i>					
NM_009253	Serpina3m	Serine (or cysteine) peptidase inhibitor, clade a, member 3k	Structural analysis of the murine orthologue of human antichymotrypsin. Contrapsin inhibits trypsin-like proteases	Down, 0.48	0.000
NM_008061	G6pc	Glucose-6-phosphatase, catalytic	Key enzyme in homeostatic regulation of blood glucose levels.	Down, 0.93	0.000
NM_008618	Mdc	Malate dehydrogenase, cytoplasmic	Malate dehydrogenase activity	Down, 0.49	0.001
NM_138745	Mthfd1	Methylenetetrahydrofolate dehydrogenase	Amino acid metabolism	Down, 0.42	0.000
NM_030721	Acox3	Acyl-Coenzyme A oxidase 3, pristanoyl	Cellular lipid metabolism, electron transport	Down, 0.40	0.000
NM_178734	D030014N22Rik	RIKEN cDNA D030014N22 gene	Regulation of cellular metabolism Involved in histone pre-mRNA processing	Up, 0.56	0.000

Table 7.2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
<i>Differentially expressed genes associated with transporters</i>					
NM_009512	Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5	Catalyze the first step in the conjugation of C24 bile acids (choloneates) to glycine and taurine before excretion into bile canaliculi by activating them to their CoA thioesters. Activate secondary bile acids entering the liver from the enterohepatic circulation	Down,0.61	0.000
<i>Differentially expressed genes associated with other functions</i>					
AK085347	D630014	Fibronectin type iii domain containing 8 E21	Hypothetical protein	Up, 0.95	0.001
NM_009725	ATP5f1	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit b, isoform 1	Oxidative phosphorylation	Down, 0.53	0.000
NM_011802	Clpx	Caseinolytic protease x	Directs the protease to specific substrates, Chaperone functions	Down, 0.45	0.000
NM_009450	Tubb2	Tubulin, beta 2	Major constituent of microtubules, Binds two moles of GTP, one at an exchangeable site on the beta chain and one at a nonexchangeable site on the alpha-chain	Down, 0.60	0.000
NM_146870	Olf25	Olfactory receptor 25	Responsible for the recognition and G protein-mediated transduction of odorant signals, Initiate a neuronal response that triggers the perception of a smell	Up, 0.51	0.000
NM_175431	A83009	RIKEN cDNA A830093I24 gene 3I24Rik	Hypothetical protein	Up, 0.42	0.000
NM_145597	BC021367	cDNA sequence BC021367	Hypothetical protein	Up,0.67	0.000
AK033851	9330101	Riken cDNA 9330101j02 gene	Hypothetical protein	Up, 1.45	0.000

j02Rik

Table 7.2 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
AK129447	9330101	mRNA for mc protein	Cellular macromolecule metabolism, protein metabolism	Up,0.68	0.000
AK046853	j02 B83002	10 days neonate medulla oblongata cDNA	Hypothetical protein	Up, 0.48	0.000
NM_017479	9I03 Myst4	MYST histone acetyltransferase monocytic leukemia 4	Transcriptional activation	Up,0.63	0.000
AK009029	230000 4M11	Nuclear receptor coactivator 2	Transcriptional coactivator for steroid receptors and nuclear receptors.	Up.0.52	0.000
NM_010753	MAD4	Max-interacting transcriptional repressor	Transcriptional repressor	Down, 0.45	0.000
NM_011153	Gsbs	G substrate	Endogenous protein phosphatase inhibitor	Up, 0.42	0.000

Table 7. 3. Differentially expressed gene list in E-TN compared to E-HS rats at the end of 26 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
<i>Differentially expressed genes associated with cell cycle and transcription</i>					
NM_019937	Cdk11	Cyclin 11	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism, cell cycle	Down, 0.49	0.000
AK016816	SOX-5	Sry-box containing gene 5	Activates transcription	Down, 0.72	0.000
NM_013684	TBP	Tata box binding protein	Functions at the core of the DNA-binding multiprotein factor TFIID	Down, 0.44	0.000
<i>Differentially expressed genes associated with immune system</i>					
NM_031162	CD3-zeta	cd3 antigen, zeta polypeptide	T-cell activation	Down, 0.60	0.001
NM_205823	Tlr12	Toll-like receptor 12	Cytokine secretion and the inflammatory response, innate immunity	Down, 0.52	0.000
U46841	Tcrb-V13T	cell receptor beta, variable 13	Cellular defense response, immune response	Down, 0.73	0.000
AK039193	A230106 M20Rik	Riken cDNA a230106m20 gene Immunoglobulin C2 type	Immunoglobulin	Down, 0.71	0.001
<i>Differentially expressed genes associated with electron transport and oxidative stress</i>					
AY339599	ND3	NADH-ubiquinone oxidoreductase chain 3	ATP synthesis coupled electron transport	Down, 0.47	0.000
AY339599	ND34	NADH-ubiquinone oxidoreductase chain 4	Generation of precursor metabolites and energy, phosphorus metabolism	Down, 0.42	0.000
AY339599	ND6	NADH-ubiquinone oxidoreductase chain 6	Oxidative phosphorylation	Down, 0.42	0.000
AY339599	CO I	Cytochrome c oxidase subunit i	Catalyzes the reduction of oxygen to water	Down, 0.42	0.000
AY339599	ATPase	ATPse subunit 8	ATP metabolism	Down, 0.42	0.000
AY339599	Cytb	Cytochrome b	Generates an electrochemical potential coupled to ATP synthesis	Down, 0.42	0.000
AY339599	COX2	Cytochrome c oxidase polypeptide 2	Component of the respiratory chain	Down, 0.42	0.000
AY339599	ATPase 6	ATP synthase f0 subunit 6	Translocation of protons across the membrane	Down, 0.42	0.000
AY339599	ND1	NADH dehydrogenase subunit 1	Electron transport	Down, 0.42	0.000

Table 7.3 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
AK053672	ND2	NADH dehydrogenase subunit 2	ATP synthesis coupled electron transport	Down, 0.66	0.001
AY339599	ND5	NADH dehydrogenase subunit 5	Oxidative phosphorylation, phosphate metabolism	Down, 0.42	0.000
AY339599	ND14L	NADH dehydrogenase subunit 4l	ATP synthesis coupled electron transport	Down, 0.42	0.000
AY339599	COX3	Cytochrome oxidase subunit 3	Oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	Down, 0.42	0.000
NM_007453	Prdx6	Peroxiredoxin 6	Protection against oxidative injury, redox regulation	Down, 0.61	0.000
<i>Differentially expressed genes associated with defense system and detoxification</i>					
NM_025569	GST	Microsomal glutathione s-transferase 3	Glutathione metabolism, metabolism of xenobiotics	Down, 0.97	0.000
NM_019552	mdr/tap	ATP-binding cassette, sub-family b	ABC transporters	Down, 1.06	0.000
NM_013873		Sulfotransferase family 4a, member 1	Sulfate conjugation of drugs	Down, 0.52	0.000
NM_009998	CYP2B10	Cytochrome p450, family 2, subfamily b, polypeptide 10	Heme-thiolate monooxygenases	Up, 0.66	0.001
<i>Differentially expressed genes associated with other functions</i>					
NM_022982	Rtn4r	Reticulon 4 receptor	Mediates axonal growth inhibition	Up, 0.45	0.000
NM_021342	Kcne4	Potassium voltage-gated channel, isk-related subfamily, gene 4	Modulates the gating kinetics and enhances stability of the channel complex	Up, 0.53	0.000
NM_134064	Rnf44	Ring finger protein 44	Biopolymer metabolism and modification, cellular macromolecule metabolism and physiological process	Up, 0.52	0.001
NM_011706	TRPV2	Transient receptor potential cation channel, subfamily v, member 2	Transduce physical stimuli in mast cells	Down, 0.49	0.000
NM_198118	9130019P16Rik	Riken cDNA 9130019p16 gene	Hypothetical protein	Down, 0.68	0.000
AK020879	EIF4E	Eukaryotic translation initiation factor 4e	Nuclear import factor	Down, 0.62	0.000
NM_013632	Pnp	Purine-nucleoside phosphorylase	Purine and pyrimidine metabolism	Down, 0.75	0.000

Table 7.3 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
NM_030238	Dhcr24	Dynein cytoplasmic 1 heavy chain 1	Cytoskeleton organization and biogenesis	Down,0.98	0.000
NM_009127	SCD-1	Stearoyl-coenzyme a desaturase 1	Important component of leptin's metabolic actions	Down,1.66	0.000
AK003868	F830021 d11Rik	Riken cDNA f830021d11 gene	Hypothetical protein	Down,0.84	0.000
NM_133979	Tmem16 k	Transmembrane protein 16k	Hypothetical protein	Down,0.58	0.000
NM_173751	ilvb	tolactate synthase	Valine, leucine and isoleucine biosynthesis	Down, 0.53	0.003
NM_021877	hr(rhsl)	Hairless	Regulates the timing of epithelial cell differentiation in both the epidermis and hair follicle	Down, 0.53	0.000
NM_139139	Dnajc17	Dna segment, chr 9,	Establishment of cellular and protein localization	Down,0.61	0.000
NM_016796	Vamp4	Vesicle-associated membrane protein 4	Remove an inhibitor (probably synaptotagmin IV) of calcium-triggered exocytosis	Down,2.73	0.000
NM_130866	Olf78	Olfactory receptor 78	Sensory perception of smell	Down, 0.62	0.000
AK030994	Rnf157	Riken cDNA 2610036e23 gene	Biopolymer metabolism, cellular macromolecule metabolism, protein metabolism	Down, 0.54	0.000
NM_173788	NPR2	Natriuretic peptide receptor 2	Receptor for atrial natriuretic peptide	Down, 0.81	0.001
NM_199449	Zhx2	Zinc fingers and homeoboxes protein 2	Transcriptional repressor	Down, 0.75	0.000
NM_021402	Ube2j2	ube6p homolog	Selective degradation of misfolded membrane proteins	Down, 0.67	0.001
NM_027055	Cyp2c70	Sperm acrosome associated 4	Hypothetical protein	Down, 0.40	0.000
NM_183137	2410002I 01Rik	Riken cDNA 2410002i01 gene	Hypothetical protein	Down, 0.72	0.000
NM_198673	Odf311	Outer dense fiber of sperm tails 3-like 1	Protein of unknown function	Down, 0.58	0.001
AK007330	Cpa1	Carboxypeptidase a1	Cellular protein metabolism	Down, 0.73	0.000
NM_007800	Ctsg	Cathepsin g	Regulate the reorganization of vimentin filaments, occurring during cell differentiation, movement and mitosis	Down, 1.00	0.000
AK015030	ubc	Ubiquitin-conjugating enzyme e2e 3	Catalyzes the covalent attachment of ubiquitin to other proteins	Down, 0.72	0.000
NM_007455	Ap1g2	Adaptor protein complex ap-1, gamma 2 subunit	Establishment of cellular localization, intracellular protein transport, endocytosis	Down, 0.95	0.000

Table 7.3 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
AK012848	Zfp618	Zinc fingerprotein 618	Hypothetical protein	Down, 0.91	0.000
NM_080793	Setd7	Lysine methyltransferase	DNA metabolism, chromosome organization and biogenesis	Down,0.47	0.001

Table 7. 4. Differentially expressed gene list in E+TN compared to E+HS rats at the end of 26 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
<i>Differentially expressed genes associated with cell cycle and transcription</i>					
NM_007631	Ccnd1	Cyclin D1	Cell division	Down, 0.65	0.000
NM_027031	1700007I06	Riken cDNA 1700007i06 gene	Cell division and chromosome partitioning	Down, 0.42	0.001
NM_138580	Rdbp	rd rna-binding protein	Negatively regulates the elongation of transcription by RNA polymerase II	Down, 0.46	0.000
<i>Differentially expressed genes associated with other functions</i>					
M86390	Msn	Moesin	Regulation of actin cytoskeleton	Down, 0.97	0.000
NM_134046	2810429O05R	RIKEN cDNA 2810429o05 gene	Hypothetical protein	Down, 0.48	0.001
NM_009918	CNG-3	Cyclic nucleotide gated channel alpha 3	Depolarization of cone photoreceptors	Down, 0.53	0.000
AK080641	B930095G15R	RIKEN cDNA b930095g15 gene	Hypothetical protein	Down, 0.57	0.000
NM_013586	Lox13	Lysyl oxidase-like 3	Oxidoreductase activity	Down, 0.65	0.000
NM_030721	Acox3	Acyl-coenzyme a oxidase 3, pristanoyl	Fatty acid metabolism	Down, 0.51	0.000
NM_016974	Dbp	D site albumin promoter binding protein	transcriptional activator for CYP2A4 and CYP2A5	Down, 0.75	0.000
AK036654	Ltbp1	Latent transforming growth factor beta binding protein 1	Critical roles in controlling and directing the activity of TGFB1 and structural role in the extra cellular matrix	Down, 0.46	0.000
AK035610	9530076L18	Hypothetical protein 9530076l18	Unknown function	Up, 0.77	0.000
NM_175539	A130007F10	Riken cDNA a130007f10 gene	Hypothetical protein	Up, 0.49	0.000
NM_026301	Rnf125	Ring finger protein 125	Positive regulator of T-cell activation, immune response	Up, 0.40	0.000

Table 7.4 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	p-value
NM_009998	CYP2B10	Cytochrome p450, family 2, subfamily b, polypeptide 10	Involved in an NADPH-dependent electron transport pathway, Oxidizes xenobiotics	Up, 0.84	0.000
NM_175126	Zcchc3	Zinc finger, cchc domain containing 3	Chaperones / Intracellular trafficking and secretion	Up, 0.47	0.000

Table 7. 5. Differentially expressed gene list in E-TN compared to E+HS rats at the end of 26 day treatment period

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
<i>Differentially expressed genes associated with immune system</i>					
AK007622	Ig lambda	Immunoglobulin lambda chain, variable 1	Immunity	Down, 0.73	0.050
NM017370	Hp	Haptoglobin	Immune function	Down, 0.49	0.110
NM022415	Ptges	Prostaglandin E synthase	Protection against harmful bacteria	Down, 0.45	0.111
NM026328	Reg4	Regenerating islet-derived family, member 4	Inflammatory and metaplastic responses of the gastrointestinal epithelium	Down, 0.43	0.127
BC054544	Ctnnd1	catenin (cadherin associated protein), delta 1	Leukocyte transendothelial migration	Down, 0.63	0.044
NM009841	Cd14	cd14 antigen	inflammatory response,	Up, 0.40	0.061
NM177853	Il2rb	interleukin 2 receptor, beta chain	Inflammatory response	Up, 0.52	0.119
AK014121	Ly6k	lymphocyte antigen 6 complex, locus k	Immune response	Up, 0.52	0.124
NM011091	Lilrb3	leukocyte immunoglobulin-like receptor, subfamily b, member 3	Humoral immune response	Up, 0.41	0.125
<i>Differentially expressed genes associated with lipid metabolism</i>					
NM009381	Thrsp	Thyroid hormone responsive spot14 homolog	Lipogenesis	Down, 0.76	0.111
NM017399	Fabp1	Fatty acid binding protein 1, liver	Intracellular lipid transport	Down, 0.75	0.108
NM007988	Fasn	Fatty acid synthase	Lipid metabolism	Down, 0.68	0.128
NM177470	Acaa2	Acetyl-coenzyme a acyltransferase 2	Fatty acid beta-oxidation cycle in the mitochondria	Down, 0.50	0.107
NM030721	Acox3	Acyl-coenzyme a oxidase 3, pristanoylprotein 1	Peroxisomal fatty acid beta-oxidation system	Down,0.54	0.050
NM146094	Fads1	Fatty acid desaturase 1	Lipid metabolism	Down,0.78	0.144
NM176840	Osbpl11	Oxysterol binding protein-like 11	Lipid metabolism, lipid transport	Down,0.47	0.128
NM_022415	Ptges	Prostaglandin e synthase	Cellular lipid metabolism	Down, 0.45	0.111
NM_008280	Lipc	Lipase, hepatic	Important enzyme in HDL metabolism	Down, 0.42	0.145
NM_011480	Srebf1	Sterol regulatory element binding factor 1	Sterol biosynthesis and the LDL receptor gene	Down, 0.53	0.134
BC031813	Cyp51	Cytochrome p450, family 51	Cholesterol biosynthesis, oxidoreductase, steroid biosynthesis	Down, 0.47	0.134

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_013495	Cpt1a	Carnitine palmitoyltransferase 1a	Fatty acid metabolism	Down, 0.66	0.141
NM_176840	Osbp11	Oxysterol binding protein-like 11	Lipid metabolism	Down, 0.47	0.128
NM_022415	Ptges	Prostaglandin e synthase	Fatty acid metabolism	Down, 0.45	0.111
NM_008512	Lrp1	Low density lipoprotein receptor-related protein 1	Lipid metabolism	Up, 0.51	0.085
NM_013873	Sult4a1	Sulfotransferase family 4a, member 1	Lipid metabolism	Up, 0.41	0.099
NM_007453	Prdx6	Peroxiredoxin 6	Protection against oxidative injury, redox regulation	Down, 0.55	0.067
AK035028	Fat3	Fat tumor suppressor homolog 3	Inhibit lipogenesis	Up, 0.45	0.114
<i>Differentially expressed genes associated with transporter proteins</i>					
NM_009674	Anxa7	Annexin a7	Calcium/phospholipid-binding protein involved in exocytosis	Down, 0.52	0.064
NM_008063	Slc37a4	Solute carrier family 37, member 4	Carbohydrate transport and metabolism	Down, 0.61	0.068
NM_007830	Dbi	Diazepam binding inhibitor	Intracellular carrier of acyl-CoA esters.	Down, 0.62	0.146
NM_009034	Rbp2	Retinol binding protein 2, cellular	Intracellular transport of retinol	Down, 0.40	0.089
U95131	Slc10a1	solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Bile acid transporter	Down, 0.44	0.144
NM_011706	Trpv2	transient receptor potential cation channel, subfamily v, member 2	Ion transporter	Down, 0.49	0.073
NM_025468	Sec113	Sec11-like 3	Protein export	Down, 0.48	0.008
NM_181422	Pkd211	polycystic kidney disease 2-like 1	Ion transport	Up, 0.58	0.134
AK051433	Fkbp4	fk506 binding protein 4	Intracellular trafficking of steroid hormone receptors	Up, 0.45	0.107
NM_010295	Gclc	Glutamate-cysteine ligase, catalytic subunit	Free radical scavenger, rate-limiting enzyme in GSH synthesis	Up, 0.43	0.110
NM_145146	Afm	Afamin precursor	Glycoprotein transport	Down, 0.67	0.064

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_026130	Srpr	Signal recognition particle receptor	Protein export	Down, 0.47	0.134
NM_007873	Doc2b	double c2, beta	Membrane trafficking	Up, 0.50	0.067
NM_029638	Abp1	amiloride binding protein 1	Oxidoreductase activity	Up, 0.45	0.083
NM_175126	Zcchc3	zinc finger, cchc domain containing 3	Intracellular protein trafficking	Up, 0.55	0.007
<i>Differentially expressed genes associated with apoptosis, cell cycle and growth</i>					
NM_009828	Ccna2	Cyclin A2	Regulate the cell cycle	Down, 0.51	0.149
NM_025840	Bzw2	Basic leucine zipper and w2 domains 2	Translation initiation factor activity	Down, 0.41	0.134
AK008900		Hypothetical protein, mgc: 11613	Play a role in cell proliferation.	Down, 0.41	0.083
NM_019639	Ubc	Ubiquitin c	Play a role in cell proliferation and apoptosis	Down, 0.53	0.067
NM_178111	Trp53inp2	Tumor protein p53 inducible nuclear protein 2	Play a role in cell proliferation.	Down, 0.44	0.110
NM_025816	Tax1bp1	Tax1 (human t-cell leukemia virus type i) binding protein 1	Negative regulation of programmed cell death	Down, 0.41	0.121
NM_008059	G0s2	g0/g1 switch gene 2	Regulate cell adhesion and cytoskeleton organization	Up, 0.41	0.153
NM_008612	Mnat1	Menage a trois 1	Involved in cell cycle control and in RNA transcription by RNA polymerase II.	Up, 0.43	0.150
NM_008368	Il2rb	Interleukin 2 receptor, beta chain	Chaperone activity	Up, 0.56	0.120
NM_010517	Igfbp4	insulin-like growth factor binding protein 4	Cell growth, cell size regulation	Down, 0.47	0.144
AK015703	Nudt12	Nucleoside diphosphate linked moiety x-type motif 12	DNA replication, repair	Down, 0.75	0.139
NM_023794	Etv5	RIKEN cDNA 8430401f14 gene	Nucleic acid metabolism	Down, 0.54	0.110
NM_022032	Perp	Perp, tp53 apoptosis effector	Programmed cell death	Down, 0.56	0.064
AK016816	Sox5	sry-box containing gene 5	Nucleic acid metabolism	Down, 0.74	0.067
NM_007907	Eef2	eukaryotic translation elongation factor 2	Protein biosynthesis	Down, 0.42	0.119
NM016898	Cd164	cd164 antigen	Fas induced apoptosis	Down, 0.52	0.124
NM_009870	Cdk4	cyclin-dependent kinase 4	Cell cycle regulation	Down, 0.69	0.016

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_007631	Ccnd1	Cyclin D1	Cell cycle transition from G1 to S phase	Down, 0.52	0.128
AK036654	Ltbp1	latent transforming growth factor beta binding protein 1	Bind with Igfbp3 to interact with TGF-beta system	Down, 0.47	0.044
NM_016690	Hnrpd1	Heterogeneous nuclear ribonucleoprotein d-like	Nucleic acid metabolism	Down, 0.56	0.067
NM_010870	Birc1e	Neuronal apoptosis inhibitory protein, related sequence 4	Negative regulation of apoptosis	Up, 0.43	0.149
AK020842	Ntng1	Netrin g1	Cellular morphogenesis during differentiation	Up, 0.65	0.139
NM_008612	Mnat1	Menage a trois 1	Ubiquitin protein ligase activity	Up, 0.43	0.149
BC057917	Syvn1	synovial apoptosis inhibitor 1, synoviolin	Negative regulation of apoptosis	Up, 0.57	0.123
NM_011264	Rev3l	Rev3-like, catalytic subunit of DNA polymerase zeta rad54 like	DNA repair, Essential role in cell survival	Up, 0.51	0.117
NM_010364	Gtf2h4	general transcription factor ii h, polypeptide 4	Nucleotide excision repair of DNA	Up, 0.47	0.143
AK122501	Sipa1l2	signal-induced proliferation-associated 1 like 2	Signal induced proliferation	Up, 0.51	0.149
<i>Differentially expressed genes associated with oxidative phosphorylation and detoxification</i>					
NM009127	Scd1	Stearoyl-coenzyme a desaturase 1	Cu,Zn-SOD activity	Down, 2.53	0.002
NM010359	Gstm3	Glutathione s-transferase, mu 3	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles	Down, 0.52	0.049
NM029555	Gstk1	Glutathione s-transferase kappa 1	Detoxification	Down,0.60	0.083
NM025569	Mgst3	Microsomal glutathione s-transferase 3	Functions as a glutathione peroxidase	Down,0.69	0.073
NM009419	Tpst2	Protein-tyrosine sulfotransferase 2	Belongs to the protein sulfotransferase family	Down,0.50	0.046
NM009155	Sepp1	Selenoprotein p, plasma, 1	extracellular antioxidant defense, transport of selenium to tissues	Down,0.50	0.111
NM017370	Hp	Haptoglobin	Hp plays a pivotal role in reducing renal oxidative damage during haemolysis	Down,0.50	0.110
NM_009725	ATP5f1	ATP synthase, h+ transporting, mitochondrial f0 complex, subunit b 1	Electron transport	Down,0.61	0.044

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_016702	Agxt	Alanine-glyoxylate aminotransferase polypeptide 1	Glyoxylate detoxification (in the peroxisomes).	Down,0.56	0.085
NM_013809	Cyp2g1	Cytochrome p450, family 2, subfamily g, polypeptide 1	Detoxification	Down,0.62	0.124
NM_007453	Prdx6	Peroxiredoxin 6	Reduce H2O2 and short chain organic, fatty acid, and phospholipid hydroperoxides	Down,0.55	0.067
NM_201644	Ugt1a9	UDP glucuronosyltransferase 1 family, polypeptide a9	Detoxification	Up, 0.58	0.073
NM_201644	Ugt1a12	UDP glycosyltransferase 1 family, polypeptide a10	Detoxification	Up, 0.47	0.073
NM_201643	Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide a6a	Detoxification	Up, 0.51	0.107
NM_010361	Gstt2	Glutathione s-transferase, theta 2	Detoxification	Up, 0.40	0.117
NM_009998	Cyp2b10	Cytochrome p450, family 2, subfamily b, polypeptide 10	Human cytochrome P450 CYP2D6	Up,0.87	0.044
NM_013873	Sult4a1	Sulfotransferase family 4a, member 1	Catalyze the sulfate conjugation of many drugs, xenobiotic compounds, hormones	Up,0.40	0.099
NM_007409	Adh1	Alcohol dehydrogenase 1	Colic acid synthesis	Up, 0.88	0.080
AK039618	Gls2	Glutaminase 2	Glutamate metabolism	Down, 0.56	0.080
NM_145079	Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide a6a	Conjugation and elimination of xenobiotics	Down, 0.51	0.107
NM_021507	Sqrdl	Sulfide quinone reductase-like	Electron transport	Down, 0.66	0.110
NM_201643	Ugt1a5	UDP glucuronosyltransferase 1 family, polypeptide a5	Conjugation and elimination of xenobiotics	Up, 0.50	0.051
NM_201645	Ugt1a12	UDP glucuronosyltransferase 1 family, polypeptide a2	Conjugation and elimination of xenobiotics	Up, 0.53	0.063
NM_201641	Ugt1a10	UDP glycosyltransferase 1 family, polypeptide a10	Conjugation and elimination of xenobiotics	Up, 0.41	0.139

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
<i>Differentially expressed genes associated with carbohydrate and protein metabolism</i>					
NM_011458	Serpina3k	Serine (or cysteine) peptidase inhibitor, clade a, member 3k	Endopeptidase inhibitor activity	Down, 0.68	0.083
NM_173751	Ilvbl	Bacterial acetolactate synthase-like	Vailne, leucine and isoleucine biosynthesis	Down, 0.62	0.119
NM_146217	Aars	Alanyl-trna synthetase	Cellular protein metabolism	Down, 0.46	0.080
NM_007800	Ctsg	Cathepsin g	Cellular protein metabolism	Down, 0.84	0.044
NM_010176	Fah	Fumarylacetoacetate hydrolase	Protein metabolism	Down, 0.42	0.145
NM_011904	Tll2	Tolloid-like 2	Protein metabolism	Down, 0.48	0.065
NM_133739	Tmem123	transmembrane protein 123	Glycoprotein	Down, 0.51	0.067
NM_009975	Csnk2b	casein kinase ii, beta subunit	Protein kinase activity	Down, 0.45	0.010
AK016729	Pfkfb2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	Carbohydrate metabolism	Down, 0.44	0.088
AK053672	Dmx12	nadh dehydrogenase subunit 2kinase 2	ATP synthesis coupled electron transport	Down, 0.93	0.009
NM_026917	Zdhhc3	zinc finger, dhhc domain containing 3	Protein metabolism	Up, 0.49	0.111
AK082919	Axin1	Axin 1	Protein catabolism	Up, 0.45	0.116
<i>Genes associated with other functions</i>					
NM_053207	Egln1	Egl nine homolog 1	Cellular oxygen sensor	Down, 0.76	0.050
AK003868	1110020k19Rik	RIKEN cDNA 1110020k19 gene	Unknown function	Down, 0.82	0.009
NM_021720	Donson	downstream neighbor of son	Hypothetical protein	Down, 0.59	0.062
AK016046	4930545h06Rik	RIKEN cDNA 4930545h06 gene	Unknown function	Down, 0.48	0.076
AK016045	4930545e07Rik	RIKEN cDNA 4930545e07 gene	Unknown function	Down, 0.46	0.124
NM_147119	Olfir632	olfactory receptor 632	G-protein coupled receptor activity	Down, 0.48	0.108
NM_023794	Etv5	RIKEN cDNA 8430401f14 gene	Nucleic acid metabolism	Down, 0.54	0.110
AK009357	Nkpd1	ntpase, kap family p-loop domain containing 1	Hypothetical protein	Down, 0.41	0.138
NM_177023	E130114p	RIKEN cDNA e130114p18 gene	Hypothetical protein	Down, 0.44	0.066

18Rik
Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
NM_033562	Derl2	Hypothetical protein, mgc: 11613	Hypothetical protein	Down, 0.42	0.098
NM_199449	Zhx2	zinc fingers and homeoboxes protein 2	Regulation of cellular metabolism	Down, 0.53	0.150
NM_028444	3110015b	RIKEN cDNA 3110015b12 gene	Kinase activity	Down, 0.87	0.044
	12Rik				
NM_145135	Rnh1	expressed sequence aw546468	Inhibitor or pancreatic RNase and angiogenin	Down, 0.60	0.50
NM_183137	2410002i0	RIKEN cDNA 2410002i01 gene	Unknown function	Down, 0.50	0.150
	1Rik				
NM_031164	F13b	coagulation factor xiii, beta subunit	Blood coagulation	Down, 0.41	0.085
AK016843	4933417e	RIKEN cDNA 4933417e11 gene	Unknown function	Down, 0.68	0.050
	11Rik				
NM_026523	Nmb	neuromedin b	Stimulate smooth muscle contraction	Down, 0.51	0.111
AK009774	2310043d	RIKEN cDNA 2310043d23 gene	Unknown function	Down, 0.41	0.080
	23Rik				
NM_026308	Rpp21	ribonuclease p 21 subunit	Endonuclease activity	Down, 0.51	0.080
NM_008125	Gjb2	gap junction membrane channel protein beta 2	Cell communication	Down, 0.41	0.134
BC022140	Fndc3a	Hypothetical protein loc213817	Hypothetical protein	Down, 0.45	0.122
NM_029269	Spp2	secreted phosphoprotein 2	Bone remodeling	Down, 0.54	0.098
NM_199149	BC024868	cDNA sequence bc024868	Hypothetical protein	Down, 0.67	0.064
AK032107	Snx19	RIKEN cDNA 3526401k03 gene	Hypothetical protein	Down, 0.55	0.110
X54327	2410081f0	RIKEN cDNA 2410081f06 gene	Unknown function	Down, 0.49	0.076
NM_016796	Vamp4	vesicle-associated membrane protein 4	Vesicle-mediated transport	Down, 0.47	0.050
NM_018811	Abhd2	abhydrolase domain containing 2	Unknown function	Down, 0.65	0.139
NM_025790	Them2	thioesterase superfamily member 2	Unknown function	Down, 0.45	0.083
NM_177809	AU04265	expressed sequence au042651	Hypothetical protein	Down, 0.55	0.063
	1				
NM_023422	Hist1h2bc	histone 1, h2bc	DNA binding	Down, 0.44	0.099
NM_177736	Lrrc61	leucine rich repeat containing 61	Hypothetical protein	Down, 0.43	0.080
NM_008148	Gp5	glycoprotein 5	Blood coagulation	Down, 0.60	0.144
AK029086	4732490b	RIKEN cDNA 4732490b19 gene	Unknown function	Down, 0.42	0.128
	19Rik				
AK012848	Zfp618	zinc fingerprotein 618	Hypothetical protein	Down, 0.53	0.112

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
BC028441	Mosc1	Moco sulphurase c-terminal domain containing 1	Hypothetical protein	Down, 0.53	0.110
NM_026728	Echdc2	Enoyl coenzyme a hydratase domain containing 2	Hypothetical protein	Down, 0.64	0.089
NM_080793	Setd7	Set domain containing (lysine methyltransferase) 7	Function unknown	Down, 0.56	0.050
NM_019694	Letm1	leucine zipper-ef-hand containing transmembrane protein 1	Mitochondrial calcium binding	Down, 0.49	0.083
AK014121	3110035B RIKEN cDNA 3110035B01 gene 01Rik		Unknown function	Up, 0.52	0.124
AK006046	1700016L RIKEN cDNA 1700016L15 gene 15Rik		Unknown function	Up, 0.41	0.149
NM_178915	A1839735 Expressed sequence ai839735		Hypothetical protein	Up, 0.46	0.102
AK081204	C030003HRIKEN cDNA C030003H22 gene 22Rik		Unknown function	Up, 0.41	0.110
AK006673	1700041I0RIKEN cDNA 1700041I07 gene 7Rik		Unknown function	Up, 0.44	0.149
AK009160	2310005A RIKEN cDNA 2310005A03 gene 03Rik		Unknown function	Up, 0.89	0.080
AK040105	A430061 RIKEN cDNA A430061O12 gene O12Rik		Unknown function	Up, 0.60	0.107
AK035028	9430076A RIKEN cDNA 9430076A06 gene 06Rik		Unknown function	Up, 0.45	0.114
NM_145597	BC021367cDNA sequence bc021367		Hypothetical protein	Up, 0.56	0.146
NM_029948	4930569K RIKEN cDNA 4930569k13 gene 13Rik		Hypothetical protein	Up, 0.49	0.134
AK006745	1700049J0RIKEN cDNA 1700049j03 gene 3Rik		Hypothetical protein	Up, 0.41	0.143
NM_181754	Gpr141	g protein-coupled receptor 141	Orphan receptor	Up, 0.55	0.044
NM_173428	Sspo	sco-spondin	Play important role in placenta	Up, 0.54	0.139
NM_172701	1810007P Riken cDNA 1810007p19 gene 19Rik		Hypothetical protein	Up, 0.43	0.128

Table 7.5 (continued)

Accession number	Gene symbol	Gene name	Gene ontology	Ratio E+TN/E-TN	FDR
AK017062	4933434P	RIKEN cDNA 4933434P08 gene 08Rik	Hypothetical protein	Up, 0.41	0.120
NM_025459	1810015C	RIKEN cDNA 1810015c04 gene 04Rik	Hypothetical protein	Up, 0.43	0.106
AK005298	2810453I0	RIKEN cDNA 2810453i06 gene 6Rik	Hypothetical protein	Up, 0.51	0.059
NM_146370	Olf47	olfactory receptor 47	Olfactory senses	Up, 0.58	0.110
NM_175431	A830093I	RIKEN cDNA a830093i24 gene 24Rik	Hypothetical protein	Up, 0.44	0.064
NM_170598	Rbm12	rna binding motif protein 12	RNA binding prrotein	Up, 0.48	0.083
NM_007623	Cbx2	chromobox homolog 2	Nucleic acid metabolism	Up, 0.49	0.080
AK082919	C430019F	RIKEN cDNA C430019F06 gene 06Rik	Unknown function	Up, 0.45	0.116
NM_175531	Mrgprb2	mas-related gpr, member b2	G-protein coupled receptor activity	Up, 0.42	0.107
NM_175424	A030004J	RIKEN cDNA a030004j04 gene 04Rik	Hypothetical protein	Up, 0.46	0.060

CHAPTER VIII

SUMMARY

The objective of the present studies was to determine the temporal effects of E+ diet, HS or their combination on physiological and genomic parameters. It is well known that HS alters the gene expression of animals, with the level of response depending on severity of HS and duration of exposure. It was hypothesized that E+ diet would alter the hepatic gene expression and its response would be more severe under HS compared to TN conditions. Furthermore, it was speculated that the genomic responses to E+ diet would be different between short- and long-term exposures, with greater adaptation occurring under long-term TN than under HS conditions.

The collection of studies reported in this thesis were designed to determine the effects of a diet containing ground endophyte-infected seed on hepatic gene expression in different thermal environments, that included thermoneutral and both short- and long-term heat stress conditions. The temporal nature of these responses was of great interest, in terms of adaptation to heat stress and fescue toxicosis. Measurements concentrated on shifts in thermoregulatory ability, blood parameters known to be affected by fescue toxicosis, hepatic gene expression, hepatic cytochrome P450 isoforms, and antioxidants.

Initially, the temporal effects of E+ diet on rat physiological parameters were evaluated under TN conditions during short (five days)- and long (26 days)-term exposures. Core temperature and general activity did not change significantly due to E+

diet during both exposures. In contrast, feed intake and FCE of E+ rats decreased within the first 24 hours of E+ diet intake, during short- and long-term exposures but the values returned to pre-treatment level by Days 9 and 5, respectively. Similarly, BW was decreased on Day 1 of treatment but exhibited partial recovery by the end of the long-term study, when the values were above pre-treatment level. Glucose, urea nitrogen, cholesterol, and ALP in the serum, and wet liver weight corrected to body weight were decreased in E+ rats during short-term treatment, but by the end of the long-term study the values returned to E- level to suggest adaptation.

The temporal effects of HS were evaluated in E- and E+ rats during short (3 days)- and long-term (21 days) exposures. Hyperthermia resulted from heat exposure, but was returned to pre-heat level in both E- and E+ rats by Days 8 and 13 respectively, suggesting adaptation of both groups to HS. Heat stress decreased activity count, as well as FCE, in both groups but E- and E+ rats exhibited complete recovery by Days 7 and 8 respectively. Feed intake was decreased within the first 24 hours of HS exposure; although partial recovery of feed intake occurred in both groups, the values never reached pre-heat level. Also, feed intake of E+ rats was always lower than for E- rats. Similarly, both E- and E+ rats exhibited partial recovery in body weight; however, the growth rate was similar between E- and E+ rats at the end of long-term heat exposure suggesting adaptation of E+ rats to HS. Serum glucose levels were decreased in E+ rats during short-term treatment, but recovered completely during long-term heat exposure suggesting adaptation of E+ rats to long-term HS. However, serum cholesterol and ALP did not change during short-term HS but decreased during long-term HS suggesting deleterious effects of long-term HS. Though HS increased serum prolactin levels in both groups, the

values were always decreased in E+ compared to E- rats. These results suggest that rats exposed to one stressor (E+ or heat) exhibited greater adaptation than those simultaneously exposed to both stressors, suggesting a greater shift from normalcy with multiple stressors.

Genomic effects of E+ diet was evaluated in rat liver at the end of two time points, i.e., short- (5 days) and long-term (26 days) TN conditions. The E+ diet intake increased the gene expression of various CYPs during short-, but not long-term exposure possibly because the rats during the long-term exposure were adapted to the toxicants. However, gene expression of antioxidant enzymes was down-regulated during both periods, making the rats susceptible to oxidative stress. Genes associated with immune response were down-regulated during short-term E+ diet intake but long-term E+ intake increased the gene expression of immune response and decreased the expression of pro-inflammatory cytokines, which once again could be an adaptive mechanism seen in E+ rats during long-term TN exposures. Similarly, cholesterol biosynthesizing genes were down-regulated during short-term but increased during long-term E+ intake. These results agree with serum cholesterol levels of E+ rats during short- and long-term E+ intake under TN conditions. Apoptotic gene expression was up-regulated and heat shock protein, cell proliferation genes and DNA repair gene expressions were down-regulated in E+ rats during both short and long-term E+ intake. Down-regulation of these genes would decrease the E+ rat's ability to repair DNA damage, resulting in cell apoptosis. Similarly, genes associated with ATP synthesis, carbohydrate and protein metabolism were down-regulated in both exposures, because of which, E+ rats may not efficiently utilize the nutrient energy, and is reflected by reduced growth rate. Short-term E+ intake

up-regulated genes coding for gluconeogenesis and protein catabolism, which help the rats to maintain appropriate glucose levels for vital functions and this could be an adaptive response to decreased caloric intake during this period. However, long-term E+ intake did not result in such gene expression reflecting the complete recovery observed in feed intake by the end of the long-term study. It is likely that the reduced antioxidant activity predisposed E+ rats to hepatic oxidative stress, and suppressed immune and chaperone activities of these animals make them further susceptible to other stressors even at TN.

Gene expression of CYP isoform was up-regulated during short-term HS but not during long-term. This result is similar to the observations made at TN. Expression of antioxidant genes were decreased in E+ rats during short-term treatment but recovered completely during long-term heat exposure suggesting partial adaptation of E+ rats to heat stress. However, antioxidant levels of these rats remained decreased compared to E- rats under TN conditions suggesting oxidative stress in E+ rats at HS and these findings are similar to the observed results in E+ cattle at HS.

Immune responsive genes were not affected during short-term HS but were down-regulated in E+ rats under long-term HS suggesting the deleterious effects of long-term heat exposure and these results are in agreement with the observations reported in E+ cattle at HS.

The E+ diet intake under short- and long-term HS decreased genes associated with cell proliferation, antioxidants, fatty acid and cholesterol metabolism compared to E+ rats under TN conditions. Short-term E+ intake during HS decreased genes coding for immune responsive, CYPs and heat shock proteins, but during long-term HS exposures

these genes were up-regulated compared to E+ rats under TN conditions. Furthermore, E+ intake under short-term HS up-regulated apoptosis genes and down-regulated ATP synthesis genes compared to E+ rat under TN conditions; however such changes in gene expression were absent during long-term HS exposures suggesting adaptation of E+ rats to long-term HS. Previous results suggested that intake of an E+ diet at TN conditions up-regulated genes associated with CYPs but decreased genes coding for chaperones, antioxidants, and immune responsiveness. The present comparison revealed that added effects of short-term HS on E+ intake further impaired the animal's defense mechanism beyond the effect at TN, thereby making the animal more susceptible to various stressors. However long-term E+ intake improved animal's immune, CYP and chaperone functions suggesting signs of adaptation to HS and E+ diet.

Finally, the combination of long-term E+ and HS down-regulated immune responsive genes compared to E- rats under TN conditions. This result suggests that HS contributed to the decreased immune response here because E+ diet under long-term TN conditions up-regulated immune responsive genes compared to the same E- rats at TN. Genes coding for antioxidants were down-regulated as a result of E+ intake, with an even greater reduction with HS, resulting in greater oxidative stress at HS. Detoxification genes were up-regulated in E+ rats as a result of long-term E+ intake and this could be an adaptive mechanism to metabolize E+ toxicants. Apoptotic gene expression was down-regulated in E+ rats at HS and these results are in contrast to what was observed under TN conditions. The possible reason for this unexpected result could be up-regulation of genes coding for chaperones, DNA repair and cell survival mechanisms along with detoxification in long-term E+ rats under HS conditions. Down-regulation of genes

coding for energy metabolism along with ATP synthesis in E+ rats under long-term HS could contribute to decreased growth and production efficiencies of E+ rats, that is characteristic of fescue toxicosis.

These results suggest that rats are better adapted to long-term E+ intake under TN than under HS conditions at both physiological and genomic levels. However, E+ rats always had impaired antioxidant activities along with decreased immune and chaperone functions (except at long-term HS), making them susceptible to various other stressors. Furthermore, HS by itself decreased antioxidant and immune functions, which might explain why HS exacerbates the clinical signs associated with fescue toxicosis, apart from hyperthermia associated with peripheral vasoconstriction.

Experiment	Short-term TN	Short-term HS		Long-term TN/HS				
	E-TN vs E+TN	E+TN vs E+HS	E-HS vs E+HS	E-TN vs E+TN	E-TN vs E-HS	E+TN vs E+HS	E-HS vs E+HS	E-TN vs E+HS
Cytochrome P450	↑	↓	↑	-	↑	↑	-	↑
Antioxidants	↓	↓	↓	↓	↓	↓	-	↓
Detoxification (Phase II)	↑	-	-	↑	-	-	-	↑
Immune response	↓	↓	-	↑	↓	↑	↓	↓
Chaperonic activity & DNA repair	↓	↓	-	↓	-	↑	-	↑
Energy metabolism	↓	↓	↓	↓	-	↓	↓	↓
ATP production	↓	↓	-	-	↓	-	-	↓
Cell proliferation	↓	↓	-	↓	↓	↓	-	↓
Apoptosis	↑	↑	-	↑	-	-	-	↓

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APPENDICES

APPENDIX 1

IMPLANTING MINI MITTERS IN RATS

Male Sprague Dawley male rats were anesthetized using a mixture of ketamine (87 mg/ml) and xylazine (13 mg/ml) administered intramuscularly at 0.1 mg/100 g BW. Then, the abdominal area was shaved with a fine hair clipper and scrubbed with iodine solution (betadione) and with alcohol. A midline incision (2 to 3 cm) was made to the outer skin layer using a scalpel. Using the blunt edge of a haemostat, the peritoneal cavity was carefully penetrated and the opening was widened enough just to allow insertion of the coater transmitter into the peritoneal cavity. After assuring the absence of any internal bleeding by the cut, the incision was sutured using a sterile, synthetic, absorbable suture [coated VICRYL/Braided (polyglactin 910) /2.0; 3.0 metric] using a cutter suturing needle. Two to three interrupted sutures were first used to close the inner layer and another 2 to 3 sutures were used to close the outer skin. The site was then scrubbed with iodine and alcohol, and the rats were monitored closely until recovered from anesthesia on a warming pad.

APPENDIX 2

RNA EXTRACTION

Take 4 ml of Buffer RLT in a 15 ml tube; add 40 μ l of beta mercapto ethanol to it.

Take tissue from – 80 freezer (Max wt of tissue shouldn't exceed 250 mg).

Add tissue sample to buffer RLT and homogenize the tissue.

Centrifuge the tissue lysate for 10 min at 4000 g.

Carefully transfer the supernatant to a new 15 ml tube by pipeting.

Add equal volume of 70% ethanol to the homogenized lysate and mix immediately by shaking vigorously.

Apply the sample to an RNeasy midi column placed in 15 ml centrifuge tube. Make sure the maximum loading volume does not exceed 4.0 ml.

Centrifuge for 5 min at 4000 g. Discard the flow through.

Add 4.0 ml of buffer RW1 to the RNeasy column. Centrifuge for 5 min at 4000g. Discard the flow through.

Add 2.5 ml of buffer RPE to the RNeasy column. Centrifuge for 2 min at 4000g. Discard the flow through.

Add 2.5 ml of buffer RPE to the RNeasy column. Centrifuge for 5 min at 4000g. Discard the flow through.

Transfer the RNeasy column to a new 15 ml collection tube. Pipet the appropriate volume of RNase free water (200 micro lit) directly onto the RNeasy silica-gel membrane.

Let it stand for 1 min. and then centrifuge for 3 min at 4000 g.

Repeat the above step again.

Go to nanodrop to check the RNA concentration.

APPENDIX 3

DNase TREATMENT FOR RNA SAMPLE

Add 0.1 volume of DNase 1 buffer to the RNA sample.

Add 2 ul of DNase 1 to it (if volume \leq 200 ul. If volume \geq 200 ul, add 4 ul).

Mix gently.

Incubate at 37 C for 30 min.

DNase Inactivation

Heat the DNase treated sample tube at 70 C for 5 min.

Take two new phase lock tubes (per sample) and spin for 1-2 min at max speed (room temp).

Transfer the DNase treated sample into first phase lock tube.

Add equal volume of phenol:chloroform:isoamyl alcohol (25: 24: 1) to the RNA sample in phase lock tube.

Immediately after adding both invert it for 4-5 times. then keep in ice and then go for second sample.

Then incubate on ice for 10 min.

Centrifuge at 14000 rpm for 5 min (at room temp).

Transfer the top layer onto the new phase lock gel tube.

To this add equal volume of chloroform. Mix well.

Incubate on ice for 10 min.

Centrifuge for 5 min at 14000 rpm (room temp).

Transfer the supernatant into new tube and go ahead with the microcon purification.

Microcon Purification

Insert microcon reservoir into the vial.

Pipette RNA soln. into sample reservoir.

Centrifuge at 10000 g for 15 min at 4 C.

Transfer the flow through into a new vial.

Add equal volume of nano water to filter and spin at 10000 rpm for 10 min at 4 C.

Transfer the filter to a new vial in upright position and spin at 1000 rpm for 3-4 min.

Go to nanodrop. Desired concentration is 1 ug/ul.

APPENDIX 4

1 - 2% AGAROSE GEL ELECTROPHORESIS

Weigh 0.5 to 1 gm of agar -- add it in conical flask.

Take 5 ml of 5% TBE and add 45 ml of nanowater. Mix the solution gently by inversion.

Add this to the agar in conical flask.

Microwave the agar solution for 1 to 2 minutes.

After little cooling add 3 ul of ethydium bromide to agar and mix well.

In the mean time, clean the gel boat and comb with distilled water and prepare 1X TBE (500 ml).

Add the gel onto the boat and let it solidify.

After solidification, add 1% TBE to the gel boat.

Take 2 ul of RNA, 8 ul of water and 3 ul of loading buffer in 0.5 ml tube and load this mixture to the well.

Add 3 ul of 100 bp DNA ladder to the left extreme well.

Connect positive (red) and negative (black) to the corresponding terminals and switch to 75 – 100 V for 30-45 min.

Visualize the gel under UV light.

APPENDIX 5

MICROARRAY SLIDE QUALITY CONTROL CHECK WITH RANDOM OLIGONUCLEOTIDES

Dilute the random 9-mers oligo 1/10 in hybridization buffer

Apply the mix to a slide prehybridized with I-block

Hybridize for 5 to 6 hours at room temperature

Wash in solution 1 by moving the rack up and down 20 times then by shaking slowly for
2 minutes

Transfer slide to another rack and wash in solution 2

Wash for one minute in ultra pure water

Centrifuge for five minutes in centrivap without using the vacuum

Scan with laser power at 100% and PMT set at 500

Reagents:

Hybridization solution: 5x SSC, 0.1% SDS, 1Mm dtt

9 mers oligonucleotides: catalog # P-21681 from Molecular Probes (Product name:

Panomer 9 random oligodeoxynucleotide, Alexa fluor 546
conjugate)

MW is 4386.6, resuspended in 1X TE at 100 ng/μl

Washing solution 1: 1X SSC and 0.05% SDS

Washing solution 2: 1X SSC

APPENDIX 6

MICRO ARRAY EXPERIMENT PROTOCOL I

Invert heat block and set it at high – max heat (On Dr. An desk).

Turn on water bath (below nano water -- set at 42 C).

Prepare I block: in 50 mL tube: 5 ml of 10X PBS + 45ml of water ➡ 3 to 4 times upside down and Microwave 30sec

➡ Add the 0.2% I-Block (0.1g of I-Block/50ml 1X PBS) ➡ Vortex

➡ Keep in water bath (42°C) for 60 min (until absolutely melt of I-Block).

Take out the Cy 3 and Cy 5 primers from freezer and thaw it in ice.

Prepare 1X SSC: (100ml, using flask) ; 5ml of 20X SSC + 95ml of water ➡ Parafilm with a small opening -- Microwave 30sec ----- Keep in water bath at 42 C for 15 – 20 min.

Take the slides out and mark with pen at back. Set slide warmer to 42 C (bn 2.5 and 3).

Keep one of the red humid chamber inside slide warmer and let it warm.

RT-Primer Mix: take 3 tubes (0.5 ml) and label with Cy3, 5 and RT and take out vial 3 and 4 -----keep on ice for thawing.

Items	Cy 3 tube	Cy 5 tube
Total RNA (5 µg)	_____ µl	_____ µl
RT primer (Vial 2)	1.0 µl	1.0 µl
Nuclease Free Water (Vial 10)	_____ µl	_____ µl

Total	11.0 μ l	11.0 μ l
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Mix the RNA-RT primer mix and microfuge briefly.

Heat to 80 C for 10 min in PCR machine (last PCR machine ---- start --- 80 C).

Immediately after PCR, transfer the tubes to ice for 2 – 3 min.

In a microtube on ice prepare reaction mix.

Reaction Mix	Each Hybridization
Water	4.1 μ l
Suprase-In™ RNase inhibitor (Vial 4)	2.1 μ l
dNTP mix (Vial 3)	2.1 μ l
10X Strategen RT Buffer	4.1 μ l
0.1M dithiothreitol	4.1 μ l
Strategen RT enzyme, 200 units/ μ l	2.1 μ l
Total volume	9.0 μ l for Cy 3 and 9 for Cy 5

Gently mix the reaction mix ---- microfuge.

Add 9 μ l of reaction mix to the 11 μ l of RNA-RT primer mix.

Incubate at 42 C for 2 hours at PCR machine (start --- 42 C).

Take SSC from water bath, pour in the red humidification chamber present in slide heater.

Take one slide, keep in humid chamber with array facing down, count 5, take 2nd slide and count from 5 to 10, 3rd slide and count from 10 to 15. continue counting until 30. then take the first slide and keep on the inverted hot block --- count 5. take out and keep on the second red humid chamber. Repeat it for all the slides. (Note: slides on SSC for 30 s and on heat block for 5 s).

Take the humid chamber with slides to UV light (all slides at once) ---- ON ----Start.

Take the plastic slide case --- put slides in it after UV ----get the I block from water bath - ---Vortex --- fill it to top-----parafilm ---- keep in hybridization oven for 1 hour.

Prepare 0.2% SDS (500ml, using flask): 10ml of 10% SDS + 490ml of water. (Room Tm.)

Washes: after one hour ---

Wash slides in [0.2% SDS](#) (Room Temp.) by plunging the rack [up and down 20 times](#) then [shaking gently for 2 minutes](#).

Wash slides in [ddH2O](#) by plunging the rack [up and down 20 times](#) then [shaking gently for 2 minutes](#).(1st wash)

Wash slides in [ddH2O](#) by plunging the rack [up and down 20 times](#) then [shaking gently for 2 minutes](#).(2nd wash)

[Centrifuge for 5 minutes](#). [The array side of slides must put an opposite direction from spin way](#).

Store away from light. Keep in slide box.

After 2 hours of RT reaction:

Stop RT reaction by adding 3.5 μ l of 0.5 M NaOH/50mM EDTA.

Incubate at 65 C for 15 min in PCR machine (Start --- 65 C)

Turn on heat block for 70 and 95 C (my desk).

Take microcon tubes with filters. Add 100 μ l of 1X TE to filters and centrifuge at top speed for 3 min.

After 65 C incubation ----- Neutralize the reaction with 5 μ l of 1 M Tris – HCl.

Add 101.5 μ l of 1X TE buffer to each Cy 3 and Cy 5 vial --- Mix and load the microcon filter --- 8 min centrifuge at 10000.

Add 5 μ l of 1X TE buffer to sample reservoir – gently tap the side of the reservoir to distribute TE across membrane surface --- keep it upside down in a new tube --- centrifuge for 2 min at 5000 rpm.

Measure the volume collected in the bottom of tube (3 to 10 μ l).

Add water (Vial 10) and adjust to a final volume of 10 μ l. Go to nanodrop.

Sample ID	Final Vol. of cDNA after Step.10	Need for H2O	Concentration	260/280
	___ μ l	___ μ l	___ ng/ μ l	___
	___ μ l	___ μ l	___ ng/ μ l	___

cDNA Hybridization and Wash

Thaw and resuspend the hybridization buffer (vial 12) by heating to 70 C for at least 10 min or until completely resuspended. Vortex to ensure that the buffer is resuspended

evenly. If doing multiple slides --- repeat heating at 70 C in between. --- Microfuge. -----
 -----While pipeting avoid bubbles. Add this at the end to hybridization mixture.

In between: Thaw vial 9 in dark..

Turn heat block and water bath to 65 C.

Turn on slide heater to 65 C (Bn 4 and 4.5) and keep hybridization chamber in it. Clean cover slip in water (in beaker) --- rinse 4 – 5 times --- keep in water beaker with water for a while.

Hybridization Mixture (Room Temp):

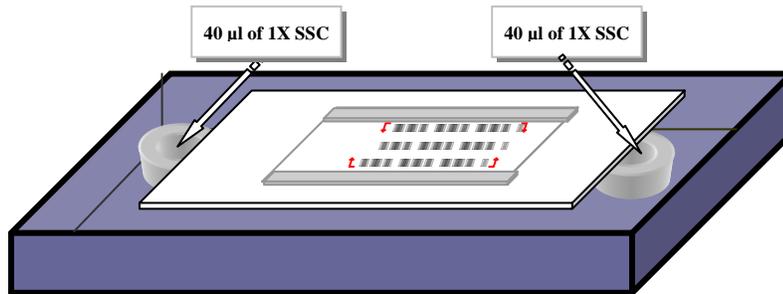
Glass Coverslip Size, mm	24x40
Final Hybridization Volume	54 μ l
Concentrated cDNA	18 μ l (9 μ l each from Cy 3 and 5)
LNA dT Blocker (Vial 9)	2 μ l
Nuclease Free Water (Vial 10)	10 μ l
2X Enhanced Hybridization Buffer (Vial 12)	24 μ l
Total Vol.	54 μ l

Gently vortex and microfuge --- incubate in PCR @ 80 C for 10 min.

Keep the slide in the hybridization chamber in the slide heater and keep cover slip on the array.

After 10 min at 80 C in PCR ----- heat the tube on heat block at 65 C until ready to load into slide (keep in block for 10 to 15 min).

Load the slide with the hybridization mix and add 24 μ l of 1X SSC at 6 -8 spots in chamber --- screw it --- water bath -65 C ---over night.



Next day morning (Protect from light ; Cover by Aluminum foil.)

Solution #1. 2X SSC, 0.2% SDS (350ml X 2); 35ml of 20X SSC + 7ml of 10% SDS + 308ml of H₂O --parafilm

Heat for 1 minutes in microwave, Keep in water bath at 65C for 10 – 15 min.

Take out Vial 1 for both Cy 3 and 5 and keep in dark for 20 min.

Turn on both heat blocks for 55 C and 70 C .

Solution #2. 2X SSC (500ml, Room Tm.); 35ml of 20X SSC + 315ml of H₂O ---parafilm

Solution #3. 0.2X SSC (500ml, Room Tm.); 3.5ml of 20X SSC + 346.5ml of H₂O---
parafilm

Take out slides from water bath on a brown paper.

APPENDIX 7

MICRO ARRAY EXPERIMENT PROTOCOL II

1. RT Reaction:

Clean the desk, pipettes and gloves with alcohol.

Take two PCR tubes, label them as reference and sample. And add the following:

	Sample	Reference
Oligo dT (5 μ l/ μ l)	1 μ l	1 μ l
PdN6 (5 μ l/ μ l)	1 μ l	1 μ l
RNA (15 μ g)	15/Conc. of RNA	15/Conc.of ref RNA
Spike	10 μ l	10 μ l
TOTAL VOL.		

Don't add spike till last. Add spike first to sample and then to reference.

2. Drying:

Take 2ml tube and keep the PCR tubes in that tube and keep in dryer. Turn it on a heat the RNA at 45C for 5-6 min. take out the tubes and measure the volume of the samples. It should be close to 15.5 μ l. If it is more again keep dry it. If the volume is too low then add sufficient amount of nano water to bring the final volume to 15.5 μ l.

3. Annealing:

Heat the PCR tubes in PCR machine.

(keep the tubes in PCR machine. Files! Start! Enter! M card! 70C-10 min. Press enter to start. After it stops, press enter and exit and open. Incubate the tubes on ice for 10 min. In mean while prepare the master mix for c DNA synthesis.

4. C DNA synthesis:

Take a PCR tube and add the following:

	X2 (one for sample and one for reference)
Water	8.8 μ l
10X buffer	6 μ l
DTT (dithioreitol)	6 μ l
25X dUTP/dNTP	2.4 μ l
RT	6 μ l
TOTAL	29.2 μ l

Remove RT reagent form the freezer only just before adding the master mix to the samples. Immediately after adding the RT keep it back in -20C.

Take half the master mix (14.6 μ l) and add in sample and the other half in the reference sample.

Incubate the tubes at 42 C for 2 hours in PCR machine. (Start, enter, 42 C for 2 hours, enter.)

5. Hydrolysis

Add the following to the tubes:

	Sample	Reference
1N NaOH	10 μ l	10 μ l
0.5M EDTA	10 μ l	10 μ l

Incubate at 65C for 15 min in PCR machine.

Add 25 μ l of 1M tris with pH of 7.4(neutralization).

Mix the contents many times by repeated pipetting.

6. Clean up

To continue with the amino allyl dye coupling procedure all Tris must be removed from the reaction to prevent the mono functional NHS ester Cy dyes form coupling to free amine groups in solution.

Microcon

Sample + nano water up to 450 μ l

Spin at 12000RPM (10000 g for 8 min).

Discard the flow through.

Repeat the above step twice using water up to 450 μ l. And again discard the flow through.

Turn the filter upside down and spin at 2000 RPM for 3 min.

Go for nano drop. : DNA-50.

Blank: nano water. The concentration of c DNA must be between 1-5% of total RNA conc.

Dry aliquot in speed vac at 45C for 30 min or till it dried up completely.

In between prepare the dyes (always keep the dyes in vacuum at 4C as the dyes are susceptible both for light and moisture.

Preparation of dyes

Take fresh tube of Cy3 and Cy5. Add 10 µl of DMSO. Mix well.

Aliquot 1.25 µl X8 tubes and immediately dry them in speed vac. And store the tubes at 4 C in vacuum.

6. Coupling:

Check the pH of 0.5 NaHCO₃ buffer (pH should be 9.0)

Add 9 µl of 0.5 NaHCO₃ to the RNA pellet. Keep it at room temperature for 10-15 min.

Now take the dye tube form 4 C. and transfer cDNA and bicarbonate buffer to the aliquot of dye. Let it incubate for 1 hour at room temperature in dark.

7. Labeling:

We do 2 hybridizations for each sample.

	Hyb1	Hyb2
Sample	Cy3	Cy5
Reference	Cy5	Cy3

8. Quenching: (Quiagen) (to remove unincorporated or quenched Cy dyes).

	Sample	Reference
Water	70 µl	70 µl
Buffer PB	500 µl	500 µl

Take 2 new tubes and fix qia quick filter column and add sample and reference to it. Spin at 12000 RPM for 30-60 sec. Discard the flow through. Add 750 μ l of buffer PE and spin for 30-60 sec. Discard the flow through. Again add 750 μ l of buffer PE and repeat the above step.

Transfer the spin unit to a new tube and add 50 μ l nano pure water to center of the filter. Let it sit at room temperature for 1 min. and then spin it at 300 RPM for 1 min. Again add 50 μ l of nano pure water and incubate at room temperature for 1 min and repeat the above step. (We should get colored cDNA in the elution tube).

Go for nano drop: see in others.

Blank: buffer EB/Water.

Note down the con, absorbance of the dyes, and the ratios.

9. Hybridization preparation:

Now combine the Cy3 and Cy5 and dry down them for 1 hour at 45-60C. cover dryer with paper to avoid the exposure of the sample to light.

In mean time prepare the hybridization solution (for each hybridization)

Water	9 μ l
20X SSC	3
HEPES	0.5
Poly A (10 μ g/ μ l)	2
Cot-1	5.5
10% SDS	0.45

TOTAL	20.45 μ L
-------	---------------

Add this solution to the dry pellet and mix well.

Heat the solution at 100C for 2 min. Let it cool for 5 min at room temperature.

Take the cassette, clean with ethanol inside. Then clean the dry kim wipe to remove all ethanol.

Set the waterbath at 65C.

Add 3X SSC to both the wells inside the cassette.

Clean the slide with air on both sides and keep it in cassette. Take a cover slip and clean on both sides with ethanol and remove dust with air on both sides.

Keep the coverslip on the array.

Check if the final volume is 20.45 μ l. If it is less then add sufficient nano water to keep the volume to 20.45 μ l.

Take 4 μ l of the sample in a pipette and add over the coverslip.

Add 10 μ l of 3X SSC on the label. Cover the top and screw the tight.

Incubate the cassette in water bath at 65C for 12 hours.

After 12 hours of incubation:

Washing:

Rinse the two glass tubes and glass bottle with distilled water thoroughly and then with nanowater.

10. Prepare the wash solution:

	Wash solution 1	Wash solution 2
Water	340 ml	350 ml

20XSSC	10	1
10% SDS	1	--

Take two slide stands and clean them thoroughly.

Take out the incubated slide form the water bath, keep it in the rack and dip the slide in WA 1 for 30-40 times. Take a 50ml tube and keep the slide in it and fill the tube with WS1. Keep in the hybridization oven for 5 min.

Then take out the slide form the tube and keep it in another rack and dip it in other WS 2 for 30-40 times. Keep the slide in another 50 ml tube and keep in hybridization oven for 5 min. then dry the slide in dryer at 600 RPM for 5 min without temp and vacuum. Take out the slide form dryer, keep it in the slide case and keep in dark place till scanning.

Pd(N)6 dilution

1 A260 unit contains: 26.5 μg .

50 units contain: $50 \times 26.5 = 1325 \mu\text{g}$.

$1325/5 \mu\text{g} = 265 \mu\text{l}$

So add 265 μl of RNase free water.

Pd(T) dilution

1 unit contain 40 μg

5 units --- 200 μg

To make 5 $\mu\text{l}/\mu\text{l}$, add $200/5 = 40 \mu\text{l}$ of water

APPENDIX 8

CELL CYCLE ANALYSIS BY PROPIDIUM IODIDE STAINING

(FLOW CYTOMETRY)

Hepatic cells trypsinized, suspended in medium + 10% FCS

Centrifuge (1000 rpm, 5 min)

Pellet suspended in 1ml PBS

After two washings in PBS, pipette cell suspension into 2.5 ml 70% Ethanol. The better way would be to add ethanol while the cell suspension is on a slow moving vortex just to avoid clustering of cells during fixation.

Incubate the cell suspension on ice for 15 min

Centrifuge the cell suspension at 1500 rpm for 5 min and discard the supernatant ethanol

Suspend the pellet in 500 micro lit propidium iodide (PI) solution in PBS {50
microgram/ml PI from 50X stock solution (2.5 mg/ml) + 0.1 mg/ml RNase A + 0.05%
Triton X-100}

Incubate for 40 min at 37 C

Add 3 ml of PBS, pellet the cells (1500 rpm, 5 min) and take off the supernatant.

Suspend the pellet in 500 microlit PBS and take for flow analysis.

VITA

Raja Sekhar Settivari was born on July 10, 1977, in Somala, Andhra Pradesh, India. He completed his high school at Kendriya Vidhyalaya, Tirupathi, India. He finished his Intermediate education at SVK educational institution at Tirupathi, India. He completed his Bachelor of Veterinary Science & Animal Husbandry degree in October, 2002 from Acharya N. G. Ranga Agricultural University, Tirupathi, India. He then moved to University of Missouri, Columbia for his Doctor of Philosophy degree in the Division of Animal Sciences and he graduated with PhD degree in December, 2007. He has taken up a research fellow position in Pediatrics at Harvard Medical School, Boston. He is married to Lakshmi Praveena Yarru and has a son, Ashrith Settivari.