EFFECTS OF FESCUE TOXICOSIS AND CHRONIC HEAT STRESS ON MURINE HEPATIC GENE EXPRESSION

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

EFFECTS OF FESCUE TOXICOSIS AND CHRONIC HEAT STRESS ON MURINE HEPATIC GENE EXPRESSION

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EFFECTS OF FESCUE TOXICOSIS AND CHRONIC HEAT STRESS ON MURINE HEPATIC GENE EXPRESSION

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ABSTRACT

Fescue toxicosis affects domestic animals grazing fescue pasture infected with the endophytic fungus, *Neotyphodium coenophialum*. Signs of fescue toxicosis include increased core body temperature and respiration rate and decreased milk yield and reproductive performance. Laboratory mice also exhibit symptoms of fescue toxicosis, as indicated by reduced growth rate and reproductive performance. Mice were used to study effects of fescue toxicosis on hepatic gene expression. Twenty-seven mice were randomly allocated to a diet containing either 50% endophyte-infected (E+; 6 ppm ergovaline) or endophyte-free (E-) fescue seed for 2 wks under thermoneutral conditions. A two-stage ANOVA of microarray data identified 36 genes differentially expressed between mice fed E+ and E- diets. The E+ diet resulted in down-regulation of genes involved in sex-steroid metabolism pathway, and genes involved in cholesterol and lipid metabolism. Genes coding for ribosomes and protein synthesis were up-regulated by the E+ diet. In a second study, mice were used to study the effects of chronic heat stress on hepatic gene expression. Twenty-five mice were randomly allocated to either chronic heat stress (cHS; 34 ± 1°C) or thermoneutral (TN; 24 ± 1°C) conditions for a period of two wks from 47 to 60 d of age. A two-stage ANOVA of 1353 gene homologous oligoarray data identified 30 genes differentially expressed between mice exposed to cHS or TN conditions. Expressions of ten genes were also measured using quantitative real-

time PCR. Genes involved in the anti-oxidant pathway and genes involved in metabolism were up-regulated due to cHS. Genes involved in generation of reactive oxygen radicals and a number of mitochondrial expressed genes were down-regulated by cHS. However, cHS did not produce an increase in oxidative stress induced mitochondrial DNA damage. Summer slump is seen in animals consuming infected-fescue forage during periods of elevated ambient temperatures (summer) to produce a range of phenotypic disorders. In the final study, effects of heat stress on changes in gene expression due to fescue toxicosis in mice liver were studied using DNA microarrays. Our goal was to characterize the differences in gene expression in liver of mice exposed to summer-type heat stress (HS) and E+ when compared to mice fed E+ at TN. Mice were fed E+ diet under HS (34 ± 1°C; n = 13; E+HS) or TN conditions (24 ± 1°C; n = 14; E+TN) for a period of two wks between 47 to 60 d of age. Forty-one genes were differentially expressed between treatment groups. Genes coding for phase I detoxification enzymes were up-regulated in E+HS mouse liver. This detoxification pathway is known to produce reactive oxidative species. We observed an up-regulation of genes involved in the protection against reactive oxidative species. Key genes involved in *de novo* lipogenesis and lipid transport were also up-regulated. Finally, genes involved in DNA damage control and unfolded protein responses were down-regulated. In summary, mice fed E+ diet at TN resulted in change in expression of genes involved in sex steroid pathway while this pathway was not perturbed in mice exposed to HS or to E+HS treatments. Changes in expression of genes involved in lipid and cholesterol metabolism pathway occurred in mice exposed to E+ and to E+HS treatment and not in mice exposed to HS only. Anti-oxidant gene expression changes occurred in mice exposed to HS and to E+HS, but not in E+ treated

mice. Interestingly, gene expression changes involved in detoxification pathway were seen only in mice exposed to combination of E+ and HS. Biological pathways and gene expression changes identified in mouse liver due to E+, HS, and E+HS might help to understand molecular mechanisms by which fescue toxicosis and heat stress affect animals.

CHAPTER I

INTRODUCTION

Fescue toxicosis is an economically important disease seen in cattle, sheep, horses and other domesticated animals. Fescue toxicosis is associated with the presence of an endophytic fungus, *Neotyphodium coenophialum*, in fescue (Bacon et al., 1977). Animals are affected when they graze fescue pasture infected with endophytic fungus, especially in summer months when ambient temperatures are high (Stuedemann et al., 1985a). Cattle consuming infected fodder show four types of disorders, which include fescue foot, bovine fat necrosis, fescue toxicosis, and summer syndrome. Fescue toxicosis is of economic importance for beef cattle production. Signs of fescue toxicosis include increased rectal temperature, respiration rate and heart rate with increased salivation; while plasma prolactin and milk production decline. Reproductive performance of the animal is also reduced (Hemken et al., 1981).

Prolonged summer heat stress affects livestock by multiple physiological mechanisms that reduce animal productivity. Across the United States, heat stress (HS) results in total losses that are estimated between $1.69 and $2.36 billion to livestock industries (St-Pierre et al., 2003). Chronic heat stress (33-35°C) in rodents has also been shown to reduce growth, increase susceptibility to disease, and compromise reproduction in males and females (Gordon, 1993).

Summer syndrome occurs during periods of elevated ambient temperature, when animals are fed endophyte-infected fescue (E+). It is characterized by hyperthermia with an accompanying decrease in feed intake and growth. Heat stress and poor body weight

gain resulting from toxicosis are most severe at the onset of high ambient temperature and humidity during the spring and summer (Hemken et al., 1981). Rectal temperature and respiration rate increased in E+ fed heifers and steers under HS conditions, but not cooler temperatures (Hemken et al., 1981). Rats on an E+ diet at thermoneutral (TN) temperature showed decreased average daily gain, feed intake and core body temperature, while feeding E+ under HS resulted in increased core body temperature compared to rats fed endophyte-free (E-) diet (Spiers et al., 2005). Laboratory mice have previously been used as a model for fescue toxicosis because these animals exhibit reduced growth, reproduction, and lactation when fed an E+ diet (Miller et al., 1994; Zavos et al., 1987). However, the molecular mechanisms by which the heat stress and fescue toxins cause fescue toxicosis in cattle, rats, and mice are not known.

Objectives

The objectives of this research were to: 1) determine gene expression changes due to fescue toxins in murine liver; 2) identify gene expression changes due to chronic heat stress in murine liver; and 3) determine the effects of heat stress in mice fed endophyte-infected fescue seeds.

CHAPTER II

LITERATURE REVIEW

Fescue Toxicosis

Introduction:

Tall fescue (*Festuca arundinacae*) is the major forage grass grown on more than 14 million ha of pasture and hay land in the eastern and northwest US (Buckner et al., 1979). This is also the most commonly used cool season grass in the southeastern US. In a study of 21 states, tall fescue was found to be used primarily for hay and pasture production with 8.5 million cattle and 688,000 horses fed (Hoveland, 1993).

From an agronomic perspective, fescue is desirable because of its ease of establishment, range of adaptation, extended grazing season, and tolerance to poor management (Stuedemann and Hoveland, 1988). A survey conducted by Shelby and Dalrymple (1987) indicates that 95% of all tall fescue pastures in USA are infected with endophyte. Bacon et al. (1977) determined that tall fescue was infected by an endophytic fungus and hypothesized that fescue toxicosis might involve the fungus *Epichloe typhina.* Later this fungus was reclassified as *Acremonium coenophialum*. Initially, several alkaloids were extracted from tall fescue, of which, some were biologically active. Studies of this fungal species proved that it is claviceps related, toxic, and possessed the potential for ergot alkaloid synthesis (Porter, 1995). Endophyte infected tall fescue has more effective insect resistance than endophyte-free tall fescue due to the production of peramine, a toxin with insect deterring activity, which is lacking in infection free tall fescue (Bacon, 1995).

Animals grazing endophyte-infected tall fescue had lower performance than expected. This was puzzling since well-managed tall fescue had high nutrient quality and should have led to good animal performance. Calf weaning weights on endophyte-infected tall fescue were 190 kg, while beef calves weigh an average of 227 kg/calf with good management practices (Hoveland, 1993). Pregnancy rate of heifers on endophyte-infected as compared with endophyte-free tall fescue pastures were reduced from 96% to 55% (Schmidt et al., 1986).

Disease associated with consumption of endophyte-infected fescue

Fescue foot:

This is a dry, gangrenous condition most often associated with lameness and the loss of ear tips and tail tips, and in acute situations, feet and hooves or the entire limb of the affected animal. This condition occurs in winter and seems to be associated with low ambient temperatures. Animal performance is reduced. The toxic compounds produced by *Acremonium coenophialum* cause vasoconstriction in the extremities, which results in thrombosis. However, even though a causal relationship between consumption of endophyte-infected (E+) fescue and fescue foot has been observed, adequate evidence is lacking to conclusively prove that this fungus is responsible for fescue foot (Schmidt and Osborn, 1993).

Bovine fat necrosis:

This condition is characterized by the presence of a mass of hard or necrotic fat in the abdominal cavity. The manifestation of fat necrosis results in inadequate physical

space within the abdominal cavity and reproductive tract. Thus, dystocia and digestive disturbances are common signs. Fat necrosis is likely to occur where the pasture sward is essentially pure fescue and these pastures have high levels of nitrogen fertilization (Stuedemann et al., 1985b); however, the exact mechanism responsible for bovine fat necrosis has not been established.

Fescue toxicosis:

This condition is also referred to as 'summer slump' or 'summer syndrome' because of unthrifty animal appearance and poor performance during summer. The signs of fescue toxicosis are reduced animal weight gains, reduced feed intake, intolerance to high environmental temperature, excessive salivation, rough hair coat which the animal fails to shed in the spring, elevated body temperature and endocrine imbalance (Hemken et al., 1979). Animal behavior is altered. Animals seeks shade, stand in water and spend less time grazing during hot parts of the day (Bond et al., 1984a; Stuedemann et al., 1985b).

Biochemical effects of fescue toxicosis

Blood chemistry:

In some experiments serum cholesterol levels differ significantly between steers that graze E+ fescue and steers that do not. It is reported that steers grazing E+ forage cultivars (Gl-307) had lower serum cholesterol concentrations than steers grazing Gl-306 (a low endophyte line), Kenly or KY-31 (cultivars of tall fescue not infected with

endophyte). This difference in serum cholesterol was accentuated during the summer months (Stuedemann et al., 1985a).

High endophyte consumption can cause serum alkaline phosphatase (AP) activities to decrease. Heifers fed high endophyte (> 90% infected) Gl-307 hay with supplemental corn and mineral mix had significantly lower AP activities than heifers fed low endophyte Kenly (< 1% infected) hay with supplemental corn and mineral mix during a period of heat stress (34 ± 1ºC). However, during a thermoneutral period (21 ± 1ºC), these heifers did not differ in AP activities (Boling et al., 1989). Alkaline phosphatase activities of steers grazing E+ fescue were significantly lower than those of steers that grazed low-endophyte fescue. These steers were subjected to average environmental temperatures that ranged from 24.7ºC to 27.7ºC (Boling et al., 1989).

Hormonal changes:

A consistent measurable result of fescue toxicosis is decreased serum prolactin. This decrease of serum prolactin is independent of environmental conditions (Elsasser and Bolt, 1987). Administration of thyrotropin releasing hormone normally stimulates prolactin secretion in livestock, but the magnitude of this release is reduced by the endophyte (Thompson et al., 1987). The effect of the endophyte upon serum prolactin was found to be less in Zebu steers compared with Angus steers (Thompson et al., 1987). Toxins reduced serum prolactin in sheep and in mares within 2 to 3 days. Ergot compounds bind to dopaminergic receptors and inhibit prolactin secretion (Goldstein et al., 1980). The dopaminergic effect of the toxins is illustrated by the increase in prolactin

secretion found following administration of dopamine antagonist to cattle and sheep (Elsasser and Bolt, 1987; Lipham et al., 1989).

Biologically, prolactin does not stimulate growth, at least in sheep. Thus, the reduced serum prolactin in E+ fed sheep is an expression of increased neural dopaminergic activity due to fescue toxicosis rather than being related to reduced growth. Administration of metaclopramide, a dopaminergic antagonist, in steers with reduced serum prolactin from grazing toxic fescue, was followed by both increases in serum prolactin and average daily gain (Lipham et al., 1989). Luteinizing hormone is of interest because this hormone is important for ovarian function. Fescue toxicosis had no effect on luteinizing hormone in steers even though serum prolactin was reduced (Thompson et al., 1987). Other hormones related to growth that have been investigated relative to fescue toxicosis include thyroid hormone, insulin, and growth hormone. Fescue toxicosis did not affect basal or glucose stimulated insulin secretion (Thompson et al., 1987). The same group found that serum growth hormone was either increased or not altered by endophyte presence. An increase could be a consequence of decreased forage intake because decreased feed intake is accompanied by an increase in serum growth hormone (Thompson and Stuedemann, 1993).

Fescue toxicosis is responsible for reduced performance

Effect on animal gain:

Early work on endophyte effects on animal performance was conducted with beef steers grazing tall fescue pastures during summer months. Decreased gains by steers grazing on endophyte-infected fescue were uniform and were not limited to geographical

location or management conditions (Paterson et al., 1995). Others have reported that reduced gains could occur at any time throughout the year (Hoveland et al., 1984).

It also has been suggested that average daily gain decreased approximately 0.045 kg for each 10% increase in fescue pasture infection rate (Schmidt and Osborn, 1993). The ergopeptide concentration in endophyte-infected pasture is greatest during May; however, signs of fescue toxicosis are most evident during July and August, suggesting an interaction with environmental temperature (Hemken et al., 1981).

Effect on milk production:

Milk production was reduced by as much as 45% in beef cows, 50% in beef heifers and 60% in dairy cows consuming fescue infected diets (Hemken et al., 1979). Milk production in dairy cows consuming E- fescue was not different from those consuming Alfa-Alfa orchard grass while, consumption of E+ fescue resulted in reduced feed intake, decreased milk yield, and loss of body weight (Strahan et al., 1987). Cows grazing E+ produced 25% less milk than cows grazing on either E- or orchard grass (Peters et al., 1992). The effect of infected-fescue on milk production appears to be more detrimental in horses than in cattle. About 88% of gravid mares grazing E+ fescue suffered from agalactia after foaling (Monroe et al., 1988). The mechanism by which milk yield is reduced in animals suffering from fescue toxicosis is not clear. Prolactin is involved in lactogenesis and mammogenesis in cattle. Synthetic ergot, bromocryptine (dopaminergic receptor agonist) was used to cause depression of serum prolactin in pre-partum cattle. This resulted in reduced milk yield, but the same treatment had no effect once lactogenesis had been established (Smith et al., 1974). Prolactin reduction pre-

partum results in decreased lactational persistence in dairy cows grazing infected-fescue compared with other forages (Seath et al., 1956). However, endophyte reduces milk yield post-partum via reduced feed intake as reviewed by Schmidt and Osborn (1993).

Effect on female reproduction:

Schmidt et al. (1986) observed calving rates of 96% for beef heifers raised on E- fescue (0 to 5% plants infected) compared to 55% for those raised on E+ fescue in which 80 to 90% of plants were infected. Only 33% of primiparous cows grazing highly infected pastures were successfully rebred compared to 93% on low endophyte pastures. The conception rates decreased 3.5% for each 10% increase in fescue infection as reviewed by Porter and Thompson (1992). A study using ewes revealed that the time to conception after introduction of the ram was lengthened on E+ pasture. Necropsy results and time to return to estrus indicated embryonic mortality as the cause (Bond et al., 1988). Puberty, as determined by the first increase in serum progesterone in Angus heifers, was delayed by E+ fescue. Using ultrasound to detect first ovulation, 62% of the heifers grazing E+ fescue had reduced serum progesterone levels indicating that corpus luteum function was sub-optimal (Estienne et al., 1990). The endophyte has more dire consequences in pregnant mares than in sheep or cattle. A survey of Kentucky horse farms indicated that 40% of mares on E+ fescue (72% endophyte infection) had reproductive abnormalities. Agalactia was the most prevalent (53%), followed by prolonged gestation (38%), abortion (18%), thickened placentas (9%) and foal loss (16%) as reviewed by Porter and Thompson (1992).

Studies were conducted using laboratory species to study the effects of E+ fescue on reproduction. When comparing laboratory and livestock species, it must be noted that prolactin is necessary for the maintenance of the corpus luteum in laboratory species (Smith, 1980), but not in livestock. This difference is important when considering pregnancy because reduced prolactin would result in loss of pregnancy in the laboratory species, but not in livestock. Mice of the CD-1 strain, used as a mouse model for fescue toxicosis, had lowered fertility when pair-fed toxic fescue seed. The female mice were more sensitive than the male to the negative effects of the endophyte (Zavos et al., 1987). In rats, a step-wise reduction in the number of rats with litters was found as the rate of dietary E+ seed increased from 20 to 40% (Varney et al., 1988). None of the rats maintained on the 40% infected diet gave birth, although 33% of the rats in this group had become pregnant. Thus, endophyte-infected seed interrupts pregnancy in rats. Mice fed either 50% endophyte-infected or non-infected fescue seed for 35 days revealed that the endophyte produced an increased incidence of abnormal sequences in estrous cycles (Zavos et al., 1988a). Feeding E+ (80%) seed based diets in both male and female rats for 60 days prior to cohabitation resulted in a significant depression in fertilization rates (Zavos et al., 1988b).

Effect on male reproduction:

In male rats, a diet of infected-fescue seed (50%) decreased daily sperm production potential, testicular parenchyma, and epididymal weight (Zavos et al., 1986). Prolactin influences pituitary gonadotropin release and the growth of male accessory reproductive glands. Bromocryptine, a dopamine agonist, decreased prolactin and

delayed testicular growth in rams (Barenton and Pelletier, 1980). Holstein bulls fed either E+ or E- fescue forage supplemented with grain from 2 to 13 months showed that endophyte had no effect on genital tract organ weight or length (Evans et al., 1989). Endophyte-infected fescue did result in a decrease in serum prolactin but was without effect on serum testosterone. There was no effect of endophyte on sperm maturity or daily sperm production potential. Endophyte-infected fescue diet reduced gonadotropin releasing hormone-stimulated testosterone in 3 month old but not in 8 or 12-month-old bulls. The number of sertoli cells in the testes was reduced by endophyte at all ages. The reduction of sertoli cells suggests permanent injury to the testicle (Alamer and Erickson, 1990).

Animal behavior:

Long before the discovery of endophyte, it was known that cattle grazing fescue spent more time in shade compared with cattle grazing on other forages. Steers grazing a high endophyte experimental fescue line spent less time grazing during the day and more time grazing during the night and had reduced total grazing time compared with steers grazing a low-endophyte fescue (Bond et al., 1984a). Steers on E- fescue spent 60% of the time between 1200 to 1800 h grazing while steers on E+ fescue spent only 4 to 6% of that time grazing (Stuedemann et al., 1985a). When heifers were offered diets in self-feeders containing 60% fescue seeds, either E- or 75% infected 11 of 12 heifers avoided diets with E+ seeds (Garner and Cornell, 1987).

Role of Ergot peptide alkaloids:

The major toxins associated with Acremonium-infected grasses and with animal toxicosis are the ergot alkaloids. Ergot alkaloids are grouped into two broad classes: the ergoline alkaloids (Ex: lysergic acid, lysergol, ergonovine) that contain the lysergic ring structure and the ergopeptine alkaloids that have a tripeptide cyclol moiety (Ex: ergovaline, ergotamine). Stuedemann et al. (1998) reported that 94% of alkaloids in cattle grazing E+ were found in urine and 6% in the bile. The authors suggested that forestomachs of ruminants (rumen, reticulum, and omasum) are the absorptive sites for ergot alkaloids. Research using nonruminants suggests that ergopeptine alkaloids are excreted via the bile and ergoline alkaloids are excreted through the urine (Hill et al., 2001).

Ergovaline and lysergic acid amides are the major ergot alkaloids in *A. coenophialum* infected tall fescue. Fescue toxicosis in animals has been reported to occur at 50 ng of ergovaline/gram of grass. The diverse number of biologically active compounds in infected-fescue would reduce the overall quantity necessary to produce fescue toxicosis (Porter, 1995). The ergovaline ring structure found in all ergot peptide alkaloids share structural similarities with norepinephrine, epinephrine, dopamine, and serotonin. Hill et al. (2001) suggested that simple ergoline alkaloids are more likely to cross digestive barriers than the ergopeptine alkaloids in ruminants and that the transport mechanism is an active process.

Other grass toxicities:

Perennial ryegrass staggers is a disorder of animals grazing perennial ryegrass pastures. The causative agents are compounds called tremorgens; the most important being lolitrem B, produced by endophytic fungus *Acremonium lolli* (Miles et al., 1992).

The disease is characterized by neurological signs such as incoordination, staggering, head shaking, collapse and animals appear to be normal until disturbed. Mortality can occur from misadventure if affected animals fall over cliffs, into ditches or ponds. Signs of ryegrass staggers develop when the lolitrem B concentrations exceed 2 to 2.5 μg/g of dry matter (Cheeke, 1995). Decreased serum prolactin is noted in lambs grazing endophyte-infected ryegrass pastures. Serum aspartate amino transferase was elevated in sheep with ryegrass staggers which suggest hepatobilary damage especially when exposed to these toxins over a long time period (Piper, 1989). A perennial ryegrass staggers problem has been reported in Oregon and northern California (Cheeke, 1995).

Resistance to fescue toxicosis

Breed differences:

Brahman cattle are known for their heat tolerance and may be better adapted to resist or tolerate hyperthermia observed during hot weather. Angus and a Brahman-Angus cross both exhibited decreased gains as a result of endophyte presence. However, the magnitude of the decrease (14 vs. 38%) was less for Brahman cross steers (McMurphy et al., 1990). Brahman (BB), Angus (AA), and reciprocal cross (AB, BA) yearling heifers and steers were tested for evidence of pancreatitis (high levels of serum amylase) while grazing either infected fescue or common Bermuda grass (Nutting et al., 1992). Yearlings of BB genotype had significant lower amylase levels than all the other breed combinations in this study, regardless of the pasture. Yearlings with at least one Angus parent had much higher amylase levels when grazing infected fescue pastures than when grazing orchard grass. However, the amylase levels for BB yearlings did not differ

between the two types of pastures. Brahman heifers had a distinct advantage in growth over Angus heifers in summer months, especially when the forage was Kentucky-31 infected fescue. Average daily gain (ADG) for Brahman exceeded that of Angus by 0.14 kg/day on Bermuda pasture and by 0.23 kg/day on endophyte-infected fescue pasture from April to October. From October to April (winter grazing) the average daily gain for Angus exceeded that of Brahman by 0.12 kg/day on Bermuda pasture and 0.19 kg/day on endophyte-infected fescue pasture (Brown et al., 1993). Because the ADG depended on season, forage type and breed, Brown et al. (1993) suggested the existence of a genotype × environment interaction. The two breeds ranked differently for ADG by season and the differences between Brahman and Angus heifers changed with forage type. In the same study, Brahman calves had higher cholesterol (CHOL; 189 ± 5 mg/dl) than AA calves (124 ± 5 mg/dl) regardless of forage type. These investigators reported that the infected fescue decreases CHOL in AA, AB, BA and in BB steers.

Within breed differences:

Within breed variation, differences in susceptibility to fescue toxicosis have not yet been identified. Hohenboken et al. (1991) were unable to differentiate Angus cows in susceptibility to fescue toxicosis based on prolactin or cholesterol levels. Gould and Hohenboken (1993) studied progeny of a sire bred in Missouri which was reputed to produce calves resistant to fescue toxicosis. Evidence was not compelling to support the existence of differences between the sire groups for susceptibility to toxicosis. However, progeny of the Missouri bull had higher feed intake and lower rectal and body surface temperature through nearly all phases of the trial.

Experiments previously been performed to develop resistance to fungal toxins using animal breeding approaches. Facial eczema (FE) is similar to fescue toxicosis and common among New Zealand cattle and sheep and can be ameliorated by selective breeding. This is caused by the ingestion of the mycotoxin sporodesmin, which is found in the spores of the ryegrass fungus *pithomyces chartarum.* Campbell et al. (1975) (as cited by Morris et al., 1989) reported large variation in susceptibility to FE as determined by gamma-glutamyltransferase activity (an indicator of liver damage). The heritability of FE susceptibility was estimated to be 0.42. Morris et al. (1989) selected performance tested rams for increased resistance to FE and reported a genetic change of 2.4% more animals resistant per year over a five year span.

Selection for fescue toxicosis in mice:

Laboratory mice suffer from reduced growth, reproduction, and lactation when fed endophyte-infected tall fescue seed and were used as a model to study genetic variation in response to tall fescue toxicosis (Zavos et al., 1986). Mice from ICR strain founders were selected for eight generations for resistance or susceptibility to impact on post-weaning gain of endophyte-infected seed in the diet (Hohenboken and Blodgett, 1997). The first two weeks post-weaning the mice were fed a 50% commercial rodent diet + 50% E- diet. Then for the next two weeks, mice were fed a 50% commercial rodent diet + 50% E+ diet. Mice having the lowest and highest reductions in average daily gain (ADG) were selected within line to become resistant (R) and susceptible (S) line parents, respectively, for the next generation. In experiment 1, on eighth generation litters, a post-weaning test was conducted (two weeks on E- diet then two weeks on E+ diet).

Hohenboken & Blodgett (1997) found that in the first two weeks on the E- diet, S mice had higher ADG than R line mice (656 ± 9 vs. 575 ± 9 mg, respectively, $P < 0.01$). In the later 2-week period on the E+ diet, the ADG of the R line was not depressed, but ADG in the S line was depressed. The activity of phase-1 biotransformation reaction enzymes was not influenced by selection but the activities of phase-2 reaction enzymes were higher in the R line than in S line mice, regardless of the diets fed (Hohenboken and Blodgett, 1997). Uridine diphosphate glucuronosyl-transferase (GRT) was higher in the R line than in the S line mice, only for the E+ diet. Glutathione-S-transferase (GST) was higher in the R line than in the S line mice, regardless of the diet fed. In experiment 2, mice were fed on E+ diet for 6 wks. The S line mice had lower ADG than the R line on the E+ diet in the first 2 weeks. In the last 4 weeks of the trial, S line mice had a higher compensatory weight gain. The R line had higher activities of GRT and GST than the S line as in experiment 1 (Hohenboken and Blodgett, 1997). These experiments showed that selection of laboratory mice for growth rate response to toxins on endophyte-infected fescue is possible. The R line mice tend to have higher activities of GST and GRT.

Mice selected for fescue toxicosis in the previous experiment were used to determine if the toxin-containing diet depressed reproduction and mature size more severely in the S than in the R line (Wagner et al., 2000). Mice from the R line tolerated a toxin-infected diet better than did the S line mice. As time progressed, line differences to the toxins moderated (the S line became less susceptible to toxins). The toxic diet had a greater depressing effect on mature body size of S than R mice, especially males. Thus, this indicated better adaptability of R than S mice to the toxin-infected diet. Twenty eight pairs per line (S or R) × diet (E+ or E−) group were cohabitated for 36 wk. The E+ diet

produced its greatest effect within the first two litters produced. Percentage changes in reproduction caused by the E+ diet for R and S pairs, respectively, were –13 and –28 for total pups born, -10 and –25 for total pups weaned, -13 and –14 for total litters produced and –30 and –42 for total litter weight weaned.

Fescue toxicosis in other species:

Unlike cattle and sheep, pregnant mares do not exhibit an increase in body temperature when they are exposed to the endophyte toxins (Monroe et al., 1988). However, horses sweat more freely than cattle and are more capable of cooling themselves. Furthermore, no incidence of 'fescue foot' has been reported in horses. Mares consuming endophyte-infected fescue have been shown to have gestation lengths in excess of the normal range of 335 to 345 days (Monroe et al., 1988). The same group has documented reduced serum progesterone levels in gravid mares consuming endophyte-infected fescue during late gestation and also an increased incidence of dystocia and agalactia.

Sheep grazing infected fescue have reduced reproductive efficiency, circulating prolactin, cholesterol, and milk production; however, they are not as severely affected as cattle (Porter and Thompson, 1992). Ewes fed endophyte-infected fescue exhibited delayed conception after introduction of the ram, but body weight gain, gestation length, average number of lambs born, lamb body weight and lamb survival were unaffected by the endophyte (Bond et al., 1988).

In a recent study, E+ diet induced gene expression changes were studied in rat livers using DNA microarrays (Settivari et al., 2006). The endophyte-infected fescue diet

resulted in the up-regulation of genes involved in detoxification (e.g. Cyp 450), while anti-oxidant genes were down-regulated by the E+ diet. Also, genes involved in energy metabolism (ATP synthase), lipid and carbohydrate metabolism and immune function were down-regulated by E+ while genes involved in stress response and proteolysis were up-regulated.

Chronic heat stress

Economic losses are incurred in livestock industries because farm animals are raised in locations and seasons outside their zone of thermal comfort (St-Pierre et al., 2003). Across the United States, heat stress results in estimated total annual economic losses to livestock industries that are between $1.69 to $2.36 billion. Of these losses, $897 to $1,500 million occurs in the dairy industry, $370 million in the beef industry, $299 to $316 million in the swine industry, and $128 to $165 million in the poultry industry. Across states, Texas, California, Oklahoma, Nebraska, and North Carolina accounted for $728 million or 43% of the total national annual losses (St-Pierre et al., 2003).

Much work has been done to identify the physiological effects of heat stress and mechanisms by which animal productivity is reduced. Heat stress reduces the expression of estrous behavior, alters follicular development, compromises oocyte competence and inhibits embryonic development (St-Pierre et al., 2003). Thus, heat stress reduces fertility in male and female dairy and beef cattle. Beef cattle suffer less from heat stress than dairy cattle due to the lower body heat production of beef cows. In sows, HS has been associated with decreased dry matter intake (DMI) and milk yield, compromised embryo

development and sow mortality (St-Pierre et al., 2003). Similarly, prolonged severe HS reduces DMI and daily gain of broiler chickens, is associated with undesirable meat qualities, and increased mortality.

Substantial work has been done in the area of the molecular biology of thermoregulation. The effects of heat stress on cellular functions include:

- Inhibition of DNA synthesis, transcription, RNA processing and translation.
- Inhibition of progression of cell cycle.
- Denaturation and misaggregation of proteins.
- Increased degradation of proteins both by proteosomal and lysosomal pathways.
- Disruption of cytoskeletal components.
- Alteration in metabolism resulting in net reduction in cellular ATP.
- Changes in membrane permeability that lead to an increase in intracellular Na^{+}, H^{+}, and Ca^{2+}.

Cells from various organisms respond to a variety of stresses by the rapid synthesis of highly conserved proteins called heat shock proteins (HSP). These proteins are essential for the survival of cells or organisms at normal and elevated temperatures (Kregel, 2002). The HSP also play a critical role in development of thermotolerance and for protection from stresses like ischemia and energy depletion (Kregel, 2002). The commonly studied and understood HSP in mammals are those with molecular masses of 60, 70, 90 and 110 kDa. The HSP70 family has been the most studied, and in addition to hyperthermia, HSP70 transcription is induced by energy depletion, hypoxia, ischemia-reperfusion, and by reactive oxygen species (ROS). The HSP70 family produces cellular

thermotolerance by preventing protein denaturation or processing denaturated proteins that are produced by hyperthermia (Kregel, 2002). Second, HSP exert their cytoprotective effect due to their anti-oxidant action by sequestering pro-oxidant free iron, thus reducing oxidative stress. Finally, HSP regulates protein turnover (Sonna et al., 2002). It has been shown that heat sensitive organs like the liver and small intestines are the first to accumulate HSP70 in response to whole body hyperthermia (Flanagan et al., 1995).

Advances in molecular biology have provided researchers with the tools to address the issue of the causal link between gene expression changes in response to heat stress and thermotolerance. Zhang et al. (2002) used a DNA microarray approach to identify gene expression changes in rat liver in response to acute heat stress (aHS; 41°C for 30 min on two consecutive days separated by 24 h). The aHS resulted in a change in the expression of 33 genes out of a total 207 genes on the array. They showed that aHS resulted in a broad induction of genes in the anti-oxidant and drug metabolism categories and transcripts involved in DNA, RNA, and protein synthesis. The authors indicated that a physiological challenge such as heat stress generates a gene expression profile in the liver that is indicative of decreased stress protein transcription and increased expression of oxidative-stress related genes (Zhang et al., 2002).

In a different study by same group, animals exposed to aHS (41°C for 30 min on two consecutive days separated by 24 h), young rats produced a small, transient increase in oxidative stress in the liver that was associated with a decline in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). No liver damage was detected in response to oxidative stress. In aged rats, a similar hyperthermia caused extensive oxidative liver injury associated with oxidative damage to DNA and to lipids (Zhang et

al., 2003). Gene expression changes in response to exertional heat injury (EHI) in humans were examined using DNA microarrays (Sonna et al., 2004). Genes involved in immune response were the single largest category affected by EHI. They also found that genes involved in cell stress response, signal transduction, metabolism/redox control and apoptosis were differentially expressed in response to EHI. This suggests that a number of functional responses to thermal stress at the level of RNA expression are conserved across species and cell types which support the use of rodent models for thermal stress studies (Sonna et al., 2004).

Most of the gene expression changes in animal models have been studied in response to aHS in which the animals were challenged for a short period of time (30 to 60 min) at 41 to 42°C. But in production agriculture, animals are exposed to chronic heat stress (cHS) conditions during which they are subjected to prolonged periods of summer heat with ambient temperatures between 33-35°C for one or two months (Jordan, 2003; St-Pierre et al., 2003). Very little is known about gene expression changes in livestock or even in rodent models in response to cHS. Horowitz et al. (2004) used DNA microarrays to identify gene expression changes in the left ventricle of the rat heart in response to heat acclimation and its association to ischemic-reperfusion cross-tolerance. The rats were heat acclimatized by continuous exposure to 34 ± 1°C for 2 d (short-term heat acclimation; STHA) or for 30 d (long-term heat acclimation; LTHA). Then these rats were then exposed for 2 h to 41°C to characterize the heat stress response before sacrificing them for gene expression analysis. Over the course of heat acclimation, genes involved in maintaining DNA integrity (damage/repair), free radical scavenging, stress regulation and effectors demonstrated a biphasic response in which the transcript steady-

state levels showed a profound elevation on STHA and then stabilized at a lower expression during LTHA (Horowitz et al., 2004). The authors stated that onset of STHA would lead to double strand DNA damage, probably due to increased ROS production. The activation of these genes is not seen in response to acute heat stress. The LTHA was associated with the expression of genes involved with adaptive, and long-lasting adaptive signaling networks. Examples of these genes include DNAJ homologs required for stabilizing the interaction of HSP70 with unfolded proteins and enhancing the protective role of HSP. Up-regulation of anti-apoptotic genes such as bcl-2 and enhanced activation of anti-oxidants were also associated with LTHA gene expression (Horowitz et al., 2004).

Schwimmer et al. (2006) studied gene expression changes in the rat hypothalamus in response to LTHA (34 ± 1°C for 30 d), STHA (34 ± 1°C for 2 d) and accompanying hypohydration using DNA microarrays. The hypothalamic transcriptome changes occurring during the process of acclimation coincided with three major categories of genomic responses:

i) abrupt transient upregulation or downregulation of genes during STHA, followed by their return to pre-acclimation levels when acclimation homeostasis has been achieved (LTHA phase).

ii) genes showing downregulation or upregulation only upon LTHA.

iii) genes showing a sustained change in their expression throughout the entire acclimation period.

The transcriptome map of genes assigned to the first category of responses emphasizes two phenomena: (1) a marked transient upregulation in transcripts confined predominantly to genes encoding voltage-gated ion channels, ion pumps, or transporters,

as well as hormone/transmitter receptors and cellular messengers, collectively points to enhanced membrane depolarization leading to the release of transmitters and neuronal excitability at this acclimation phase; (2) a transient downregulation among the groups of genes participating in intracellular protein trafficking, metabolism, or phosphorylation processes, implies a perturbation in cellular maintenance. In contrast to the STHA phase, they observed a decrease in the expression of specific LTHA-activated genes related to various metabolic activities, including those linked with mitochondrial energy metabolism and cellular-maintenance processes, together with the resumption of preacclimation transcript levels of genes encoding proteins linked with ion movement and membrane/cellular signaling. The third category includes constitutive downregulation of genes associated with energy metabolism and food intake and the marked upregulation of a large group of genes linked with the immune response (Schwimmer et al., 2006).

Interactions of fescue toxins and summer heat stress

Cattle consuming endophyte-infected fescue often exhibit hyperthermia as shown by an increase in rectal temperature. It has also been shown that the consumption of infected fescue has the most detrimental effect on the animal when the ambient temperature exceeds 31ºC (Hemken et al., 1979). Rectal temperatures are not affected in horses consuming E+ diets (Monroe et al., 1988). Steers were fed diets composed of E- or E+ hay and seeds in a controlled environment at 21ºC and 32ºC; the main effects of endophyte presence were that feed intake and heart rate were depressed, and rectal temperature and respiration rate were elevated (Osborn et al., 1992). Intake of the E+ diet

was reduced by 36% in the cool environment, but rectal temperature and respiration rate were not affected. In the hot environment, feed intake was reduced 60% in steers fed the E+ diet and rectal temperature and respiration rate were increased due to the E+ diet. The observed hyperthermia has been suggested to be a result of peripheral vasoconstriction, which is caused by the toxins (Walls and Jacobson, 1970). Vasoconstriction decreases the animal's ability to dissipate body heat through evaporative and non-evaporative mechanisms. Increased respiration rate associated with fescue toxicosis is generally associated with increased rectal temperatures. Some animals pant in an attempt to cool themselves and dissipate the increased heat load (Hemken et al., 1981). When ambient temperature is high and the signs of fescue toxicosis are intense, animal performance is affected not only by the consumption of E+ fescue, but also by improper functioning of the animal's thermoregulatory mechanism (Schmidt and Osborn, 1993).

Al-Haidary et al. (2001) observed that consumption of an E+ diet during continuous heat challenge resulted in a marked increase in core body temperature, especially during night time exposure to heat stress, due primarily to a reduction in cutaneous heat transfer, with no effect on heat production. The E+ diet caused a reduction in blood flow to the skin of heat-challenged steers, as evidenced by the reduction in the blood flow to skin covering the inner hind limb (Rhodes et al., 1991). The authors hypothesized that the reduced blood flow to the periphery could reduce the ability to move heat from the core tissue to the surface for dissipation and result in increased rectal temperature. Holstein calves on an E+ diet during constant heat challenge (32ºC and 50% relative humidity) led to a 50% reduction in skin vaporization and no changes in heat production or respiratory vaporization (Aldrich et al., 1993). Repeated heat challenge and

consumption of an E+ diet resulted in a reduced response to E+, suggesting that heat adaptation or adaptation to E+ reduces its adverse effects (Al-Haidary et al., 2001).

Effects of fescue toxins and heat stress were evaluated using rats as an experimental model. Ergovaline, the primary toxin found in tall fescue, was injected into rats exposed to cold (7 to 9ºC) and hot (31 to 33ºC) conditions to determine effects on thermoregulatory ability. Metabolic heat production and body temperature were reduced following ergovaline injection at the cold temperature. Peripheral heat loss was decreased at the hot temperature, which resulted in increased body temperature. Therefore, thermoregulatory response to ergovaline is highly dependent on the magnitude of thermal stress (Spiers et al., 1995).

CHAPTER III

EFFECTS OF FESCUE TOXICOSIS ON HEPATIC GENE EXPRESSION

Abstract

Fescue toxicosis affects wild and domestic animals grazing fescue pasture infected with the endophytic fungus, *Neotyphodium coenophialum*. Signs of fescue toxicosis include increased core body temperature and respiration rate and decreased milk yield and reproductive performance. Laboratory mice also exhibit symptoms of fescue toxicosis, as indicated by reduced growth rate and reproductive performance. Mice were used to study effects of fescue toxicosis on hepatic gene expression. Twenty-seven mice were randomly allocated to a diet containing either 50% endophyte-infected (E+; 6 ppm ergovaline) or endophyte-free (E-) fescue seed for 2 wk under thermoneutral conditions. Liver genes differentially expressed due to fescue toxicosis were identified using DNA microarray. A two-stage ANOVA of microarray data identified 36 differentially expressed genes between mice fed E+ and E- diets. Another analysis method, Significance Analysis of Microarray, identified nine genes as differentially expressed between treatment groups and some genes overlapped with genes identified by ANOVA. Hierarchical clustering of 36 genes identified by ANOVA clearly separated the mice by diet, with 100% confidence as computed by bootstrap analysis. Expressions of 11 genes were verified using quantitative real-time PCR. The E+ diet resulted in down-regulation of genes involved in sex-steroid metabolism pathway, and genes involved in cholesterol

and lipid metabolism. Genes coding for ribosomes and protein synthesis were up-regulated by the E+ diet. Genes identified in the present analysis indicate some of the mechanisms by which fescue toxicosis occurs in animals.

Introduction

Fescue toxicosis occurs in cattle consuming forage infected with the endophytic fungus, *Neotyphodium coenophialum*, and causes annual losses in excess of $600 million to the beef industry (Hoveland, 1993). Fescue toxicosis leads to reduced feed intake and decreased milk yield, reproduction, and weight gain (Paterson et al., 1995; Schmidt and Osborn, 1993; Thompson and Stuedemann, 1993). Grazing endophyte-infected tall fescue decreases serum prolactin, cholesterol, progesterone, and LH in cattle (Burke et al., 2001). The major toxins associated with endophyte-infected grasses and with fescue toxicosis are ergopeptine alkaloids, primarily ergovaline and lysergic acid amides.

Laboratory mice were used previously as a model for fescue toxicosis because these animals exhibit reduced growth, reproduction, and lactation when fed endophyte-infected tall fescue seed (Godfrey et al., 1994; Miller et al., 1994; Zavos et al., 1987). However, the molecular mechanisms by which the toxins affect animals are not clear. Mice selected for sensitivity to fescue toxins (Hohenboken and Blodgett, 1997) were used in this study with the objective of identifying some of the molecular pathways by which endophyte-infected seeds may cause fescue toxicosis. In mammals, the liver plays an important role in detoxification and metabolism of toxins, and is a prime target of tissue injury in response to various physiological challenges. Consequently, we use microarray-based expression profiling to study the transcriptional response of mouse liver genes to the ergopeptine alkaloids.

Materials and Methods

Animal work

Mice used in this study were developed at Virginia Polytechnic Institute and State University (Blacksburg, VA) by Hohenboken and Blodgett (1997). Briefly, mice from the ICR strain (Harlan Sprague Dawley, Indianapolis, IN) were divergently selected for eight generations on an index reflecting the impact of a diet containing endophyte-infected fescue seed on growth rate following weaning. Two lines of mice were produced, one designated sensitive had slow growth on the endophyte diet, the other, resistant, had rapid growth. Only mice from the sensitive line were used in the present experiment for gene expression analysis.

In the present study, pups from 11 litters were weaned at 21 d of age. Twenty-seven mice (14 male, 13 female) were randomly allocated to receive diets containing 50% endophyte-infected (E+; Seed Research of Oregon, Inc., Corvallis, OR) or endophyte-free fescue seed (E-; Missouri Southern Seed, Rolla, MO) for a period of 2 wk from 47 to 60 d of age under thermoneutral conditions (24 ± 1ºC). Fescue seeds and rodent chow (Formulab Diet # 5008, PMI Feeds, St. Louis, MO) were ground to pass a 1-mm screen and both components were mixed in equal parts.

All mice were housed in individual cages with relative humidity maintained at 35 to 50% and a 12:12 light-dark cycle with lights on at 0700. All mice were weighed on d 1, 7, and 14 of the experiment. At the end of the experiment, mice were euthanized with carbon dioxide gas followed by cervical dislocation. Liver tissues obtained from all mice

were weighed and snap frozen in liquid nitrogen. The Animal Care and Use Committee, University of Missouri-Columbia, approved all procedures and protocols.

RNA extraction

Extraction of RNA was done by using the RNAqueous-Midi kit according to manufacturer instructions (Ambion Inc., Austin, Texas). Concentration of RNA was determined by using microcon-30 filters according to manufacturer instructions (Millipore Corp., Bedford, MA). The RNA obtained was DNase-treated by using DNA-Free kit (Ambion Inc., Austin, Texas) to remove contaminating DNA. The DNase-treated RNA was then treated with phenol: choloroform: isoamyl alcohol (25:24:1) in a 'phase lock' tube (Eppendorf, Hamburg, Germany). Concentrated RNA was checked for quality and integrity using agarose gel electrophoresis. The RNA was quantified by taking optical density (OD) readings on an ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The DNase-treated RNA samples were kept at -80ºC freezer until used.

Microarray preparation

Microarrays were prepared by printing 1,353 oligos, 50 mers in length, representing rat genes expressed in liver on Pan epoxy glass slides (MWG Biotech AG, Ebersberg, Germany). The arrays also contained 10 arabidopsis genes, each of them printed 10 times across the array as external controls (Spot report oligo^{TM}, Stratagene, Cedar Creek, Texas). Blank spots and 3x SSC were used as negative controls. Mouse Cot-1 (Applied Genetics laboratories, Inc., Melbourne, FL) and poly-A DNA were used

to detect non-specific hybridization. A robotic microarray printer with 16 printing tips was used to spot the oligos. The rat liver oligo arrays have a total of 1,920 spots and are organized in 16 blocks arrayed in four rows and four columns. Each block has 120 spots arranged in 10 rows and 12 columns. Slides were stored in the dark until used.

Microarray protocol

Samples of RNA from mice in the study and from a reference RNA pool (Universal Mouse Reference RNA, Stratagene, La Jolla, CA) were used in hybridization. This Universal Mouse Reference RNA is a pool of RNA extracted from 11 different mouse cell lines. Sample or reference RNA (15 µg) containing 10 different arabidopsis mRNA added at varying concentrations as external controls were primed using 1 µL oligo dT (10 µg/µL; IDT DNA, Coralville, IA), and 1 µL random hexamers (10 µg/µL; IDT DNA, Coralville, IA) at 70˚C for 10 min and then chilled on ice for 10 min. The cDNA synthesis was done by adding 3.0 µL (50 units/µL) of Stratascript Reverse Transcriptase (StrataScript™ RT, Stratagene, La Jolla, CA), 3.0 µL of 10x Stratascript RT buffer, 3.0 µL of dithiothreitol (0.1 M), 0.6 µL of 50x amino-allyl dUTP (Sigma chemicals, St. Louis, MO) and 5 µL of DNase and RNase-free water. The reverse transcription was done at 42ºC for 2 h. Degradation and hydrolysis of RNA was done by adding 10 µL of 1 N NaOH, and 10 µL of 0.5 M EDTA to the mix, then the reaction was incubated at 65ºC for 15 min followed by neutralization with 25 µL of 1 M TRIS, pH 7.4. The cDNA obtained was purified using microcon-30 filters (Millipore Corp., Bedford, MA) and dried in a centrivap concentrator (Labconco Corp., Kansas City, MO). Dried cDNA was resuspended in 10 µL of 0.5 M sodium bicarbonate buffer, pH 9.0, at room

temperature for 10 to 15 min. The cDNA concentrations were quantified by using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Labeling of cDNA was done by indirect labeling with Cy5 or Cy3 dyes (Amersham Biosciences, UK). The Cy3 and Cy5 dyes were prepared by adding 10 µL of DMSO to 1 mg of Cy dye and then aliquoting 1.5 µL of mix into a new tube, dried in centrivap (Labconco Corp., Kansas City, MO) and stored for future use. The labeling reaction was carried out for 1 h at room temperature in dark conditions. The removal of unincorporated dyes and concentration of labeled cDNA was done by using a Qia-quick PCR purification kit (Qiagen, Valencia, CA).

Microarray hybridization and washing

The sample and reference labeled cDNA were mixed and dried in a centrivap (Labconco Corp., Kansas City, MO) at 42ºC. The dried mix was then resuspended in a hybridization buffer, containing 2.0 µL of poly-A (10 µg/µL; IDT DNA, Coralville, IA), 6.25 µL of mouse Cot-1 DNA (3.2 µg/µL; Applied Genetics Laboratories, Inc., Melbourne, FL), 3.0 µL of 20x SSC, 0.45 µL of 10% SDS, 0.5 µL of 1X HEPES (pH: 7.0), 8.25 µL of water, and hybridized to the arrays for 12 to 14 h at 65ºC in a humid hybridization chamber. This temperature of hybridization was chosen to ensure high specificity hybridization of mouse RNA on rat oligonucleotides. After hybridization, slides were washed in solution 1 (340 mL water, 10 mL 20x SSC, 1 mL 10% SDS) for 5 min, washed in solution 2 (350 mL water, 1 mL 20x SSC) for 5 min, and then centrifuged for 5 min to dry the slide. Extracted RNA from each of the six (3 male, 3 female) randomly selected E+ mice and seven (4 male, 3 female) E- mice treatment groups were

individually hybridized with reference RNA to the array in a reference microarray design. In a reference design, each experimental sample is hybridized against a common reference RNA sample (Churchill, 2002). Two or three replicates (arrays) were done per animal out of which one was done in a dye swap design. A total of 35 arrays were done across all the animals and treatment groups.

Microarray scanning and data acquisition

Slides were scanned using an Axon GenePix 4000B scanner (Axon Instruments Inc., Union City, CA) at 5 micron resolution and the image was labeled and stored in the BioArray Software Environment database (Saal et al., 2002). Gridding was done using GenePix Pro 4.0.1.12 software (Axon Instruments Inc., Union City, CA). Data files were linked to the appropriate image file and then stored in the BASE database. The raw data files on which this paper is based have been deposited with National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with series number is GSE2134 and platform number GLP1786.

Statistical analyses of microarray data

Microarray data were filtered in the BASE database to remove control spots, 3x SSC, and blank spots. The probe intensities from each array were treated as statistically independent by the ANOVA model (Kerr and Churchill, 2001; Wolfinger et al., 2001). The log base 2 intensities for each array spot were analyzed using a mixed model approach consisting of two steps, following the procedures of Kerr and Churchill (2001)

and Wolfinger et al. (2001). The first step involved normalization across genes and the second step involved normalization within gene. The normalized data were then analyzed in a gene-specific analysis to test the effect of E+ vs E- diets on expression of individual genes. The normalization model in the first step was:

$$Y_{agp} = \mu + A_a + P_p + (AP)_{ap} + \varepsilon_{agp} \qquad [1]$$

Where Y_{agp} represents the Log 2 of the observed fluorescent intensity signal from each gene on the array; μ is the overall mean value; A_a is the main effect of array a; P_p is the main effect of printing pin p (one of the 16 pins used to print oligos on the array); $(AP)_{ap}$ is the effect of pin p within array a; ε_{agp} is the stochastic error. The residual (R_{agp}) from the step 1 was obtained by subtracting the fitted values for the effects from the Y_{agp} values. Array and pin accounted for 25% of the variance in the data. The second step of the statistical analysis consisted of gene-specific models for the residuals (R_{agp}) obtained from the normalization approach discussed above. These models were:

$$R_{dgnt} = \mu_g + (GD)_{gd} + (GT)_{gt} + (GA_n(T))_{gnt} + \varepsilon_{dgnt} \qquad [2]$$

or

$$R_{dgn} = \mu_g + (GA_n)_{gn} + (GD)_{gd} + \varepsilon_{dgn} \qquad [3]$$

Where μ_g is the mean value for gene g; $(GD)_{gd}$ is the gene g by dye d interaction on array a; $(GT)_{gt}$ is the interaction term for gene g by treatment t (these values were used to do gene-specific t-tests and to find differentially expressed genes between

treatment groups); $(GA_n(T))_{gnt}$ is a random effect for animal A_n within treatment t by gene g; $(GA_n)_{gn}$ in equation 3 is the random effect for gene g by animal A_n; ε_{dgnt} and ε_{dgn} are the stochastic errors obtained from the gene-specific models. These analyses were computed by using the Mixed Procedure of SAS (SAS Inst. Inc., Cary, NC). Analyses were done using equal and unequal variances between treatment groups to obtain lists of differentially expressed genes. The $(GA_n)_{gn}$ values obtained for genes from equation 3 were used in a Significance Analysis of Microarray (SAM; Tusher et al., 2001). Gene lists from the three analyses (Table 1) were used to identify differentially expressed genes due to the E+ vs E- treatment, and later to select genes for quantitative real-time PCR analysis (qPCR). The R_{agp} values obtained from the stage 1 normalization model were also used in a non-parametric Wilcoxon test. The probability values obtained from these analyses were used to find genes that had significantly different expression between treatment groups. Hierarchical clustering and support tree analyses of differentially expressed genes were done using TIGR multiexperiment viewer (TMEV) software (Saeed et al., 2003). Gene ontology of the genes shown in Tables 3 and 4 were obtained using DAVID (Dennis et al., 2003) and Locuslink (http://www.ncbi.nlm.nih.gov/LocusLink/) from NCBI.

Quantitative real time-PCR

Liver RNA extracted from E+ and E- mice was used in these experiments. Genes for qPCR were chosen based on the gene lists obtained using ANOVA, a non-parametric Wilcoxon test, the *P*-values of genes, and the false discovery rate (FDR) of genes obtained from SAM analysis. Of 11 gene expression profiles measured using qPCR, five

were done using Taqman probes and the remainder by SYBR green assay. From each mouse, 10 µg of total RNA was reverse transcribed using Stratascript RT (Stratagene, La Jolla, CA) with oligo dT and random hexamer primers. Then, 1.25 ng of cDNA was added to a 25 µL PCR reaction to get a final concentration of 0.05 ng/µL of cDNA. For the Taqman assay, final concentrations of forward and reverse primer were 300 nM and the probe concentration was 200 nM. For the SYBR green assay, forward and reverse primer final concentrations were 100 nM. List of forward and reverse primer sequences are shown in Table 2. Reactions were performed by using Brilliant QPCR Plus Core Reagent kit and Brilliant SYBR Green QPCR Master Mix for Taqman and SYBR Green assays, respectively (Stratagene, La Jolla, CA). Primers were designed using Primer Express (Applied Biosystems, Foster City, CA), with an annealing temperature of 60°C and amplification size of less than 150 bp. Glyceraldehyde-3-phosphate dehydrogenase (Gapd) and 18S ribosomal (Rn18s) genes were each evaluated for use as an endogenous control by comparing their expression across 4 different E+ and E- samples at three different template concentrations per sample and selecting the gene which had the lower SD and CV. Glyceraldehyde-3-phosphate dehydrogenase (Gapd) was chosen as the endogenous control gene in our experiments as it had a lower SD (0.47 to 0.80) and lower CV (0.02 to 0.04) across different samples and template concentrations compared to the Rn18s gene (SD 1.01 to 1.40 and CV 0.06 to 0.08). Relative quantification of gene expression changes were recorded after normalizing for Gapd expression, computed by using the $2^{-\Delta\Delta CT}$ method (user manual #2, ABI Prism 7700 SDS) and qPCR was done in an ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). In the $2^{-\Delta\Delta CT}$ analysis, the threshold cycle (C_T) from E- mice was used as a calibrator

sample. The threshold cycle was determined manually for each gene across all samples such that it falls in the exponential phase of PCR (user manual #2, ABI Prism 7700 SDS). Dependent variables, C_T and delta C_T values for the individual genes in E+ and E- mice were fitted to a model including effects of gender, endophyte, and their interactions using GLM of SAS (SAS Inst. Inc., Cary, NC).

Results

Animal performance

Mice fed the E+ diet gained less weight ($P < 0.01$) than those fed E- from d 1 to 7 of the experiment (0.13 and 0.85 ± 0.11 g for E+ and E-, respectively). Mice consuming E+ gained 0.78 g from d 7 to 14 compared with 1.01 (± 0.11) g for mice consuming E- ($P = 0.16$). Overall, BW gain from d 1 to 14 on the E+ diet (0.91 ± 0.15 g) was less ($P < 0.01$) than on the E- diet (1.85 ± 0.16).

Microarray experiments

Microarray analyses were conducted to find differential gene expression between E+ or E- fed mice. These analyses revealed nine and 13 differentially expressed genes using ANOVA with equal and unequal group variances, respectively, using $P \leq 0.02$ between treatment groups as the cutoff for detecting differential expression. Significance Analysis of Microarrays done on $(GA_n)_{gn}$ values obtained from the second stage ANOVA model identified nine genes as down-regulated in E+ mice compared to E- mice at a FDR of less than 40%. Genes identified using ANOVA with equal, unequal group variance and SAM were compared to identify differentially expressed genes (Table 1).

Microarray analyses, using ANOVA with unequal group variances, identified 36 genes as differentially expressed when $P \leq 0.05$ was used as a cutoff for significance between treatment groups. The E+ diet resulted in upregulation of 13 genes, while 23 genes were down-regulated (Tables 3, 4). The hierarchical clustering of these 36 genes was able to separate the animals into two groups that precisely reflect the E+ or E- treatments with 100% support from the bootstrap test. The genes were also separated into two groups of up-regulated or down-regulated genes with 100% support (Figure 1).

Microarray analyses identified genes involved in cholesterol metabolism like Atp5b, Abcb4, Scd1, Aldh1, and Aldh2 to be differentially expressed in response to E+ diet. Furthermore, genes involved in sex steroid metabolism like Hsd3b5 and Hsd17b2 were down-regulated in response to E+ diet in mice liver.

Quantitative real-time PCR

Expression of 11 genes were measured using qPCR in fourteen E+ and twelve E- mice liver samples. Among these samples, only six E+ and seven E- mice were used to generate microarray data. Nine of the 11 genes were present on the microarray and qPCR results of seven of these genes were in agreement with microarray results in terms of direction of change and magnitude. Four of the seven genes (Atpb5, Hsd17b2, Hsd3b5, expressed sequence AU018778) had concordance in terms of statistical significance obtained using a two-way student's t-test ($P < 0.05$). When Scd1 expression was measured by qPCR on the samples hybridized to the array (n = 13), both methods agreed on the direction of change (34% up-regulation) in E+ samples. However, when Scd1 expression was measured on all animals (n = 26), it showed that Scd1 was down-

regulated in the E+ group by 38% compared to the control ($P < 0.05$). For genes Abcb4 and mt-Atp8, qPCR had the same direction of fold change as in the microarray experiment (Abcb4, down-regulation in E+ group with microarray = 29%, qPCR = 13%; mt-Atp8, down-regulation in E+ group with microarray = 23%, qPCR = 7%), but differences were not detected between treatment groups ($P = 0.70$, $P = 0.41$, for Abcb4 and mt-Atp8, respectively). Two of the genes (IL-6 and Rbbp7) showed gene expression changes in the opposite direction to that of microarray results (Figure 2). One of the genes, IL-6, was expressed at very low levels and amplimer was not detected until cycle 33, even when the template concentration was increased to 1 ng/µL. There was an effect of gender for gene Hsd3b5, with 81% down-regulation in females compared to males, while gene Hsd17b2 was up-regulated in female mice by 45% ($P < 0.01$) compared to males. There was an interaction of gender and diet for Hsd3b5, where E+ resulted in 93% reduction in Hsd3b5 expression in females when compared to male mice fed E+ diet ($P < 0.01$). There was no interaction of gender and diet ($P = 0.10$) for gene Hsd17b2. The greatest difference in gene expression detected by qPCR was for Hsd3b5, which was 86% down-regulated in E+ fed animals. Expression of two genes in which probes were not present on the arrays was analyzed using qPCR. The expression of the steroidogenic acute regulatory protein (Star) gene, which controls transport of cholesterol into mitochondria for conversion into different kinds of steroids, was not different between the E+ and E- groups. Expression of Hsd3b3 was down-regulated due to the E+ diet by 50% compared to E- control mice ($P = 0.01$). Means and SE of cycle threshold obtained from qPCR between E+ and E- mice along with the *P*-values are shown in Table

Discussion

We present new findings about differential gene expression in the liver of mice in response to consumption of endophyte-infected fescue diet. Fescue toxicosis is associated with changes in expression of genes involved in carbohydrate, cholesterol, and lipid metabolism.

Carbohydrate metabolism

Janssen et al. (2000) described metabolic changes in a dietary subacute toxicity experiment with the ergot alkaloid α-ergocryptine in Sprague-Dawley rats. Total plasma cholesterol and HDL-cholesterol were decreased in a dose-dependent manner in females, and both triglyceride and glucose concentrations were decreased in the highest dose groups in both sexes. The ergot alkaloid α-ergocryptine also influenced carbohydrate metabolism in their study, with increases in insulin, glucagon, and observed glycogen storage in liver. The phosphorylase kinase, gamma 2 (testis/liver), codes for phosphorylase kinase. This important regulatory enzyme of glycogen metabolism was down-regulated in E+ mice by 34% compared to E- mice (Table 4). Mutations and deficiency of this gene cause increased glycogen storage in liver of human and rat, and is one of the conditions seen in rats fed the ergopeptine alkaloids, ergocryptine (Janssen et al., 2000) or ergometrine (Peters-Volleberg et al., 1996). Thus, downregulation of the phosphorylase kinase, gamma 2 (testis/liver) could possibly induce some of the carbohydrate metabolism perturbations after ingestion of ergopeptine alkaloids.

Cholesterol trafficking

The expression of ATP synthase H+ transporting gene (Atp5b) was increased by 100% (qPCR) in mice fed the E+ diet when compared to the control. The beta chain of Atp5b is present on the ectopic surface of the hepatocyte cell and can act as a receptor subject to stimulation by apoA I, and triggers endocytosis of high-density lipoproteins (HDL) particles by an energy dependent process (Martinez et al., 2003). In liver, HDL endocytosis by the hepatocytes is the main transport mechanism of cholesterol import from peripheral tissues. Cholesterol concentration in serum is also known to decrease in animals fed endophyte-infected fescue (Bond et al., 1984b; Nihsen et al., 2004; Stuedemann et al., 1985). The up-regulation of Atp5b might be part of a feedback mechanism in the hepatocytes. The cells, sensing a decreased serum level of cholesterol, might be trying to compensate by increasing HDL endocytosis through an increase in HDL receptor protein expression. The Paraoxinase 1 (PON1) gene was down-regulated by 20% in the E+ when compared to E- mice. The protein PON1 is known to be quantitatively associated with HDL. Furthermore, overexpressing PON1 in transgenic mice protected HDL integrity and function (Oda et al., 2002).

The expression of the stearoyl-CoA desaturase 1 (Scd1) gene was down-regulated due to E+ diet by 38% when measured in all animals (n = 26) by qPCR (Figure 2 and Table 5). The Scd1 catalyzes a rate-limiting step in the synthesis of monounsaturated fatty acids and plays an important role in fat metabolism (Ntambi, 1999). It is one of three genes induced more than 1.5-fold in response to oxidative stress and heat shock in different human cell types (Murray et al., 2004). This gene is also heavily regulated by the diet. For example, cholesterol and polyunsaturated fatty acids up-regulate Scd1, while

a diet rich in carbohydrate down-regulate it (Ntambi, 1999; Ntambi and Miyazaki, 2004). In liver, the majority of fatty acid metabolism is directed towards triglyceride synthesis and exocytosis. Changes in the expression of Scd1 are thus very likely to affect the fatty acid composition of the cells. This gene is needed for the synthesis of oleic acid, itself required for the esterification of cholesterol in the liver. Esterified cholesterol is then ready for export to the peripheral tissues in the form of very low-density lipoprotein (VLDL). A decrease in Scd1 expression would likely translate into a decrease in VLDL synthesis and cholesterol export out of liver. Therefore, it seems that mice fed endophyte-infected fescue are trying to keep cholesterol inside the liver and limit its availability to the peripheral tissues.

The aldehyde dehydrogenase family 1, subfamily A1 and A2 genes were up-regulated by 70 to 100% in E+ mice and these genes are involved in aldehyde dehydrogenase activity, bile acid synthesis, fatty acid, and glycerolipid metabolism. The ATP-binding cassette sub-family B (MDR/TAP) member 4 (Abcb4) and gene expressed sequence AU018778, which belongs to type-B carboxylesterase/lipase family, are involved in lipid transport and metabolism, respectively. The Abcb4 gene was down-regulated by 29%, while AU018778 was down-regulated by 35% due to the E+ diet when compared to the control, indicating cholesterol and lipid metabolism changes in the liver.

The disagreement of results obtained from microarray and qPCR for genes IL-6, Rbbp7 could be due to low levels of expression of these genes. Array probes with low intensities have been shown to yield greater technical variance, thus increasing the chance of being mistakenly identified as differentially expressed (Quackenbush, 2002). Thus, the low expression of IL-6 could yield a false microarray result. Another reason for

the discrepancy between microarray and qPCR results for IL-6 and Rbbp7 is that each technique may detect different splicing variants. Because the sequences of the oligos on the array are not known, we cannot rule out this possibility.

Sex steroid hormone metabolism

Expression of three of the genes affected by the E+ diet play a role in sex steroid hormone metabolism. A flowchart showing the gene expression changes in the sex steroid metabolism pathway is shown in Figure 3. The Hsd3b5 gene product catalyzes the formation of the relatively inactive steroid androstanediol from active dihydrotestosterone (DHT), and was reported to have sex-specific expression only in male mice (Abbaszade et al., 1995). In the present study, expression of this gene was also detected in female mice and was down-regulated in E+ mice by 49% compared to E- treated mice. This would reduce the conversion of testosterone and DHT to the less active androstanediol. The steroid activating isoform, Hsd3b3, which catalyzes the reverse reaction of Hsd3b5/b4, was also down-regulated in E+ mice by 50% compared to E- mice. This enzyme is essential to sex steroid hormone synthesis, as it catalyzes production of progesterone from pregnenolone, androstenedione from dehydroepiandrosterone (DHEA) and testosterone from androstenediol (Wong and Sarjeet, 2002). It is interesting to note that earlier reports indicate that consumption of an E+ diet caused a decrease in circulating progesterone levels (Estienne et al., 1990). The third gene, Hsd17b2, was down-regulated in E+ mice by 34% compared to E- treated mice. This enzyme catalyzes conversion of the highly active 17B-hydroxyl forms of sex steroids into less potent 17-keto steroids such as estradiol into estrone, testosterone into

androstenedione, and DHT into 5α- androstenedione, respectively. This enzyme is likely involved in the rapid degradation and excretion of steroids in surface epithelial cells and hepatocytes in the intestine and liver, respectively (Mustonen et al., 1998).

We studied expression of the Star gene, which controls the importation of cholesterol into mitochondria for conversion into sex steroids. Expression of Star was not different between treatment groups indicating that movement of cholesterol into mitochondria was not affected. Changes in steroid gene expression indicate that local production of progesterone and testosterone in liver is likely decreased and that the formation of inactive steroids from these hormones is also down-regulated. The amount of these hormones synthesized by the liver is likely small compared to what the gonads are producing, thus an endocrine role for the steroids produced by liver is unlikely. However, mounting evidence suggests a possible local role of steroids production in organs not traditionally associated with reproduction. For example, the P450scc and P450C17 genes are expressed in liver, brain, kidney, stomach, and duodenum of rat (Dalla Valle et al., 2002; Vianello et al., 1997) or mice (Choudhary et al., 2003). Furthermore, the P459arom gene is expressed in bone, brain, vascular tissue, adipose tissue, skin, and fetal liver of humans (Bulun et al., 2004), and in liver, pituitary, muscle, kidney, and adipose tissues of sheep (Sharma et al., 2004).The function of these proteins in these tissues is unknown but is likely important to the animals, especially as related to the conservation of expression throughout related species.

We do not know if metabolic intermediates such as pregnenolone or DHEA are likely to accumulate because we did not measure expression of P450scc and CYP17α genes. These results agree with previous studies indicating that cattle fed endophyte-

infected fescue had reduced levels of estradiol, progesterone, and LH in circulation, and in CL (Browning et al., 1998; Burke et al., 2001).

Other differentially expressed genes

The probasin gene was found to be down-regulated due to the E+ diet. This protein belongs to the lipocalpin family and expression of this gene is androgen-regulated (Rennie et al., 1993). Two genes, small inducible cytokine A6 and complement component C6 were differentially expressed and are involved in chemokine activity, immune response, and complement activation. Genes involved in apoptosis, like Caspase 6, and structural protein like crystalline-mu, were differentially expressed. The "structural maintenance of chromosome 1-like 1 protein (SMC-protein)" gene was down-regulated in E+ mice. This gene is involved in chromosome cohesion during the cell cycle and in DNA repair and has chromatin, ATP binding and ATP-binding cassette (ABC) transporter activity.

Table 1. Comparison of results from three methods used to analyze gene expression data from microarrays of liver from mice fed endophyte-infected (E+) versus endophyte-free (E-) diets

		Genes identified using [a] equal group variance at $P \leq 0.02$		Genes identified using [b] unequal group variance at $P \leq 0.02$		Significant analysis of microarray	
Gene name	Symbol [c]	Rank [d]	*P*-value	Rank [d]	*P*-value	Rank [d]	FDR [e] %
ATP synthase 8, Mitochondria	mt-Atp8		-	3	0.004	5	16 %
ATP synthase, H+ transporting	Atp5b	4	0.005	5	0.005		-
ATP-binding cassette 4	Abcb4	5	0.006	4	0.004	8	41 %
Carboxy peptidase B2	Cpb2		-		-	6	16 %
Caspase 6	Casp6		-	10	0.01	7	28 %
Complement factor c6	C6		-	9	0.009		-
Crystalline, mu	Crym	9	0.018	12	0.018		-
Cytoplasmic-gamma isoform of actin	Actg1		-	8	0.008		-
Expressed sequence AU018778	-	2	0.002		-	3	16 %
Hydroxysteroid dehydrogenase-5, Delta<5>-3-beta	Hsd3b5	1	0.002	1	0.002	2	16 %
Hydroxysteroid 17-b dehydrogenase 2	Hsd17b2	8	0.012	11	0.015	4	16 %
Interleukin 6	IL-6		-	2	0.004	1	16 %
Probasin	Pbsn	6	0.006	6	0.005		-
Retinoblastoma binding protein7	Rbbp7	3	0.003	7	0.006		-
Small inducible cytokine A6 precursor	Ccl6	7	0.011		-		-

[a] genes determined to be differentially expressed when analyzed by ANOVA with equal group variance along with ranking of genes according to the *P*-values. Empty cells indicate that the gene was not detected using particular analysis method.
[b] genes determined to be differentially expressed when analyzed by ANOVA with unequal group variance along with rankings of genes and *P*-values.
[c] Available: http://www.ncbi.nlm.nih.gov/entrez.

[d] Rank of genes identified by each method according to the *P*-values from lowest to highest.
[e] False discovery rate (FDR) % of differentially expressed genes obtained using significant analysis of microarray**.**

Table 2. Primer sequences (5'→ 3') used in real-time PCR (accession numbers are listed in Table 3 and 4)

Gene Name	Symbol	Forward [a]	Reverse [b]	Probe [c]	Product [d] size, bp
Retinoblastoma binding protein 7	Rbbp7	GCCTGAATGTGTGGGATTTAAGT	GCAGTGTGCCCTCCATGAAT	AGAAGATGGGCCTCCAGAGC [t] TCCTGT	103
Hydroxysteroid dehydrogenase-5, delta<5>-3-beta	Hsd3b5	TCGAAAACATGAAGAGGAATTGTC	AGGCTCTCTTCAGGCATTGG	AGCTGCAGACAAAGGCCA [t] AGGTGAGA	98
ATP-binding Cassette 4	Abcb4	GGAAGTCACAGTGAGCTGATGAA	CTTCTGACAGGATCTGGCTTCCT	AAGGGATCTACTTCAGACTC [t] GTTAACATGCAGACA	88
Expressed sequence AU018778	-	GGTGTTCCATCGGTGATTGTG	TCTGCATGGTCTCCTACTACATTCTT	ATCAATACTACCCAAGCTTC [t] TCATCACCCCA	131
ATP synthase, H+ transporting	Atp5b	CACCACCAAGAAGGGATCGA	GCAGGGTCAGTCAGGTCATCA	CCTCGGTGCAGGCTATCTA [t] TGTG CCTG	72
ATP synthase 8, Mitochondria	Mt-ATP8	GCCACAACTAGATACATC AACA TGATT	GGTTGTTAGTGATTTTGGTGA AGGT	none (SYBR)	130
Interleukin-6	IL-6	ACACATGTTCTCTGGGAA ATCGT	AAGTGCATCATCGTTGTTCAT ACA	none (SYBR)	84
Steroidogenic acute regulatory protein	Star	CCGGAGCAGAGTGGTGTCA	GCCAGTGGATGAAGCACC	none (SYBR)	65
Hydroxysteroid(17-B) dehydrogenase 2	Hsd17b2	AAGATGCTCACAGCAAAG TCACA	GACCCCGGCATTGTTAACC	none (SYBR)	74
Hydroxysteroid dehydrogenase-3, delta<5>-3-beta	Hsd3b3	TTGTTGGTGCAGGAGAAA GATCT	CCAACACTGTCACCTTGATGC T	none (SYBR)	115
Stearoyl-Coenzyme A desaturase 1	Scd1	TTCTTGCGATACACTCTGG TGC	CGGGATTGAATGTTCTTG TCGT	none (SBYR)	98
Glyceraldehyde -3-phosphate dehydrogenase	Gapd	TGAAGCAGGCATCTGAGG G	CGAAGGTGGAAGAGTGGGAG	none (SYBR)	102

[a] Forward = Forward primer.
[b] Reverse = Reverse primer.
[c] Probe with sequences [t] real-time PCR was done using Taqman method while for other genes SYBR green method was used.
[d] Product size, bp includes the sequences of forward and reverse primers.

Table 3. List of up-regulated genes in livers from mice fed the endophyte-infected (E+) diet obtained from microarray data analyzed by ANOVA using unequal group variance ($P \leq 0.05$)

Accession [a] Number	Symbol	Gene name		Ratio (E+/E-) [c]	*P*-values
AK010314	Atp5b	ATP synthase, H+ transporting mitochondrial F1	ATP binding, ATP biosynthesis and transport.	1.71	0.008
Y00225	Rpl3	Ribosomal protein L3.	Structural component of Ribosome, protein biosynthesis.	1.68	0.05
L04280	Rpl12	Ribosomal protein L12.	RNA, protein binding, protein biosynthesis.	1.93	0.05
M73436	Rps4x	Ribosomal protein S4, X-linked.	RNA binding, cell proliferation, protein biosynthesis.	1.64	0.02
BC023248	Actg1	Actin, gamma, cytoplasmic 1.	Motor activity, Structural component of cytoskeleton.	1.84	0.008
AK088075	H3f3b	H3 histone, family 3B.	DNA binding, nuclear, chromosomal location.	1.18	0.04
Af458413	Fmo5	Flavin containing monooxygenase 5.	Monooxygenase, oxidoreductase activity, electron	1.66	0.04
AK005822	Oplah	5-oxoprolinase (ATP-hydrolysing).	Catalytic, hydrolase activity, glutathione metabolism.	1.31	0.04
M74570	Aldh1a1	Aldehyde dehydrogenase family 1, subfamily	Aldehyde dehydrogenase activity and retinoic	2.02	0.04
AK078553	Aldh1a2	Aldehyde dehydrogenase family 1, subfamily	Aldehyde dehydrogenase activity and retinoic	1.68	0.03
BC007474	Scd1	Stearoyl-Coenzyme A desaturase 1.	Stearoyl-CoA 9-desaturase activity, fatty acid	2.07	0.02
XM_487419	-	G protein gamma-5 subunit.	Signal transducer activity, G-protein coupled receptor	1.43	0.04
BC062097	D3Jfr1	DNA segment, Chr 3, MJeffers 1.	Regulation of transcription, DNA-dependent, DNA	1.88	0.05

[a] Available: http://www.ncbi.nlm.nih.gov/entrez.
[b] Ontology classification of genes according to biological process, molecular function and cell location.
[c] Ratio (E+/E-) for up-regulated genes computed from the expression of gene in mice fed endophyte-infected (E+) diet over its expression in mice fed the endophyte-free (E-) diet.

Table 4. List of down-regulated genes in livers from mice fed the endophyte-infected (E+) diet obtained from microarray data analyzed by ANOVA with unequal group variance ($P \leq 0.05$)

Accession [a] number	Symbol	Gene name	Ontology [b]	Ratio (E+/E-) [c]	*P*-values
X54542	Il6	Interleukin 6.	Cytokine activity, acute phase, immune response.	0.66	0.004
AB042523	mt-ATP8	ATP synthase F0 subunit 8.	H+ exporting ATPase activity, phosphorylative mechanism.	0.67	0.004
AK076016	Rbbp7	Retinoblastoma binding protein 7.	Protein binding, transcriptional repressor activity.	0.79	0.006
AF164524	Cpb2	Carboxypeptidase B2 (plasma).	Carboxypeptidase activity, proteolysis and peptidolysis.	0.48	0.02
AU018778	AU018778	Expressed sequence AU018778.	Catalytic activity, hydrolase activity.	0.65	0.009
AF047600	Smc1l1	SMC (structural maintenance of chromosomes 1)-like 1.	ATP binding, ATPase activity, DNA repair, cell cycle.	0.64	0.04
X72711	Recc1	Replication factor C 1.	ATP, DNA binding, regulation of transcription-DNA dependent.	0.66	0.05
L41519	Hsd3b5	Hydroxysteroid dehydrogenase-5, delta<5>-3-beta.	C21-steroid hormone biosynthesis.	0.51	0.002
AK004950	Hsd17b2	Hydroxysteroid (17-beta) dehydrogenase 2.	Estradiol 17-beta-dehydrogenase activity, steroid biosynthesis.	0.66	0.01
J03398	Abcb4	ATP-binding cassette, sub-family B (MDR/TAP), member 4.	ATP binding, ATPase activity lipid metabolism, transport.	0.71	0.004
U32684	Pon1	Paraoxinase 1.	HDL binding, cholesterol metabolism, response to toxin.	0.79	0.05

Accession [a] number	Symbol	Gene name	Ontology [b]	Ratio (E+/E-) [c]	*P*-values
AK082479	Mlycd	Malonyl-CoA decarboxylase.	Methylmalonyl-CoA decarboxylase activity, acetyl-CoA metabolism, fatty acid biosynthesis.	0.84	0.03
AK004890	Gcgr	Glucagon receptor.	G-protein coupled receptor activity, G-protein coupled receptor.	0.81	0.05
AK005277	Phkg2	Phosphorylase kinase, gamma 2 (testis).	ATP binding, protein kinase activity, glycogen biosynthesis.	0.66	0.02
U15209	Ccl9	Chemokine (C-C motif) ligand 9.	Chemokine activity, immune response.	0.80	0.03
AF184900	C6	Complement component 6.	Complement activation.	0.64	0.04
AF005204	Pbsn	Probasin.	Odorant binding, transporter activity.	0.50	0.005
D49729	Avpr1a	Arginine vasopressin receptor 1A.	G-protein coupled, vasopressin receptor activity.	0.87	0.05
AK028544	Bteb1	Basic transcription element binding protein 1.	DNA, Nucleic acid binding, regulation of transcription, DNA-dependent.	0.71	0.03
AK011710	Casp6	Caspase 6.	Caspase activity, induction of apoptosis.	0.69	0.01
AK021042	Mak10	MAK10 homolog, amino acid N-acetyltransferase subunit.	Unknown.	0.81	0.03
AY330868	Cul4b	Cullin 4B.	Unknown.	0.71	0.05
Y17328	Crym	Crystallin, mu.	Structural constituent of eye lens.	0.76	0.01

[a] Accession number: ww.ncbi.nlm.nih.gov/entrez.
[b] Ontology is classification of genes according to their biological process, molecular function and cellular location.
[c] Ratio (E+/E-) is computed from the expression of gene in mice fed endophyte-infected (E+) diet over its expression in mice fed the endophyte-free (E-) diet.

Table 5. Threshold cycle (C_T) means (± SE) for target and Gapd genes in livers from mice fed endophyte-infected (E+, n = 14) and endophyte-free (E-, n = 12) diet obtained from real-time quantitative PCR

Symbol [a]	E+ Target Gene C_T [b]	E+ Gapd C_T [c]	E- Target gene C_T [d]	E- Gapd C_T [e]	*P*-values
Abcb4 [t]	25.7 ± 0.18	23.5 ± 0.16	25.8 ± 0.15	23.8 ± 0.40	0.70
Atp5b [t]	20.7 ± 0.12	23.2 ± 0.17	20.9 ± 0.17	22.4 ± 0.18	0.001
AU018778 [t]	22.6 ± 0.13	23.0 ± 0.14	22.5 ± 0.42	23.5 ± 0.61	0.04
Hsd3b5 [t]	27.4 ± 0.62	24.0 ± 0.25	25.3 ± 0.39	24.7 ± 0.79	0.01
Rbbp [t]	26.6 ± 0.40	23.3 ± 0.45	27.4 ± 0.43	23.6 ± 0.54	0.10
Hsd17b2	22.0 ± 0.15	18.4 ± 0.15	21.5 ± 0.19	18.4 ± 0.17	0.008
Hsd3b3	22.7 ± 0.24	18.4 ± 0.13	21.8 ± 0.24	18.5 ± 0.15	0.01
IL-6	33.3 ± 0.21	19.0 ± 0.15	33.4 ± 0.27	18.8 ± 0.18	0.45
mt-ATP8	15.1 ± 0.17	19.3 ± 0.13	15.0 ± 0.25	19.3 ± 0.17	0.41
Scd1	17.8 ± 0.31	18.4 ± 0.12	17.3 ± 0.26	18.6 ± 0.19	0.03
Star	29.1 ± 0.17	18.8 ± 0.14	29.3 ± 0.18	18.9 ± 0.20	0.95

[a] Symbol: available at www.ncbi.nlm.nih.gov/entrez. For symbol [t], real-time PCR was done using Taqman method and other genes were done by SYBR green method.
[b] E+ Target Gene C_T is cycle threshold for target gene in mice fed endophyte-infected (E+) diet.
[c] E+ Gapd C_T is cycle threshold for control gene Gapd in mice fed endophyte-infected (E+) diet.
[d] E- Target gene C_T is cycle threshold for target gene in mice fed endophyte-free (E-) diet.
[e] E- Gapd C_T is cycle threshold for control gene Gapd in mice fed endophyte-free (E-) diet.

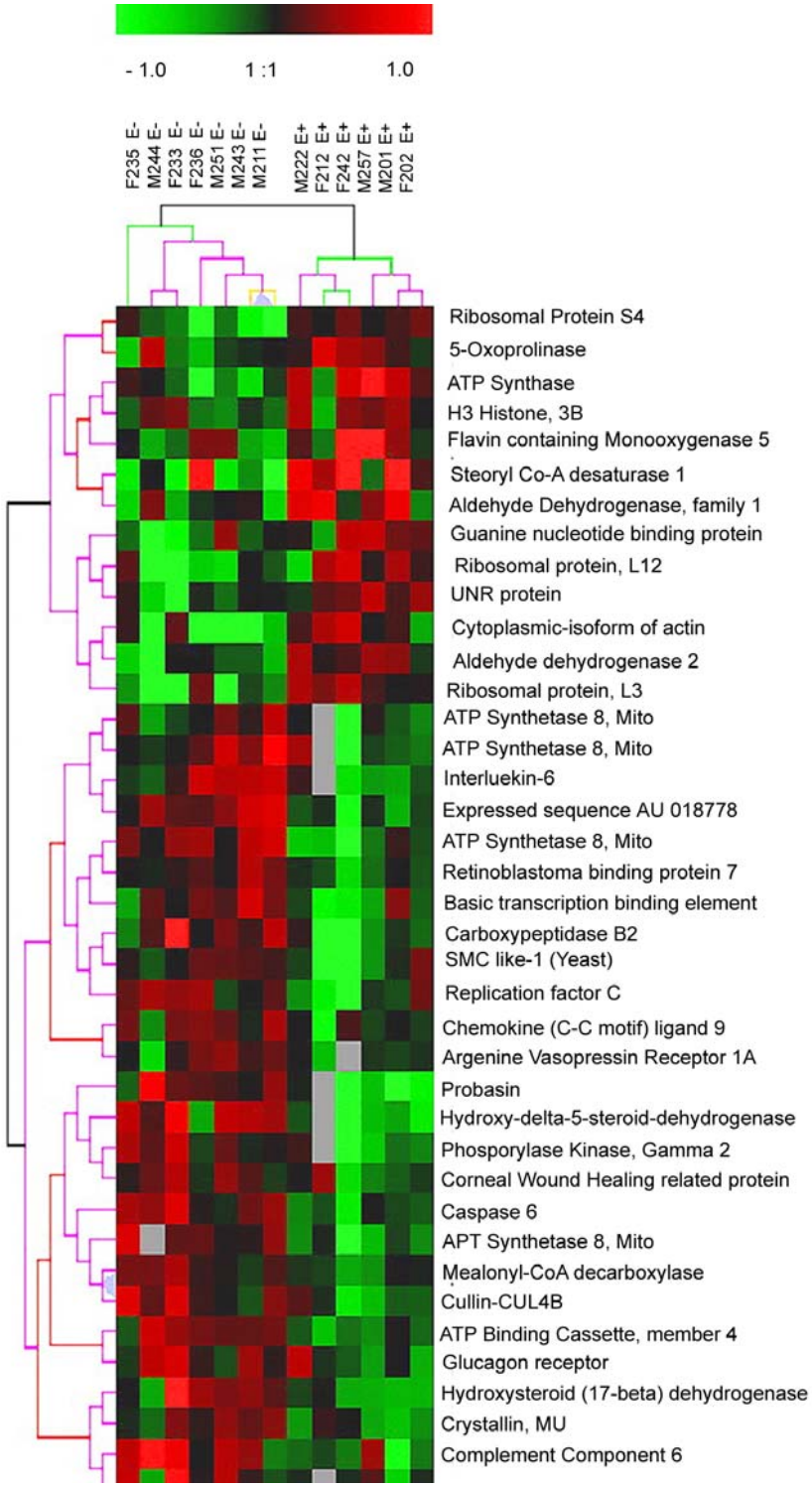

Figure 1. Differentially expressed genes ($P \leq 0.05$) and mice clustered using TIGR multi-experiment viewer (TMEV). The bootstrap analysis was done with 400 iterations to assess the significance of the hierarchical trees. The mice are divided into two groups with 100% support based on their treatment (top clustering) and 13 genes were up-regulated while 25 genes were down-regulated (clustering shown at right) by endophyte-infected (E+) treatment diet.

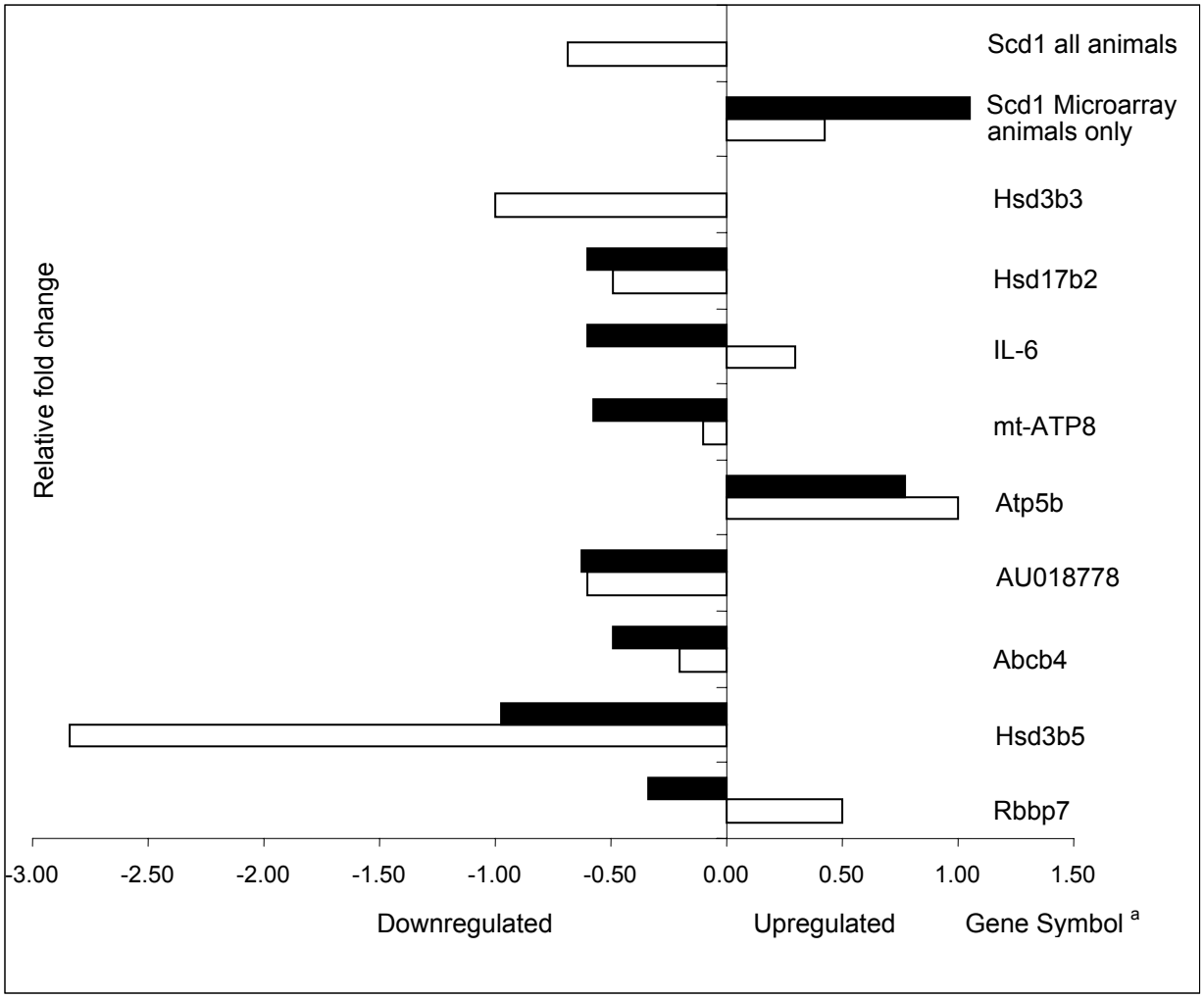

Figure 2: Expression analyses of 10 genes using oligonucleotide microarrays and quantitative PCR (qPCR). Showing log 2 of the ratio computed from the expression of gene in mice fed endophyte-infected (E+) diet over its expression in mice fed the endophyte-free (E-) diet. Solid and open bars represent microarray or qPCR data, respectively. The 'Scd1 all animals' indicate qPCR measured in 14 E+, 12 E- mice while 'Scd1 microarray animals only' indicate qPCR measured only in mice on which microarray was done (n = 13). [a] Additional information about gene symbols can be obtained from www.ncbi.nlm.nih.gov/entrez.

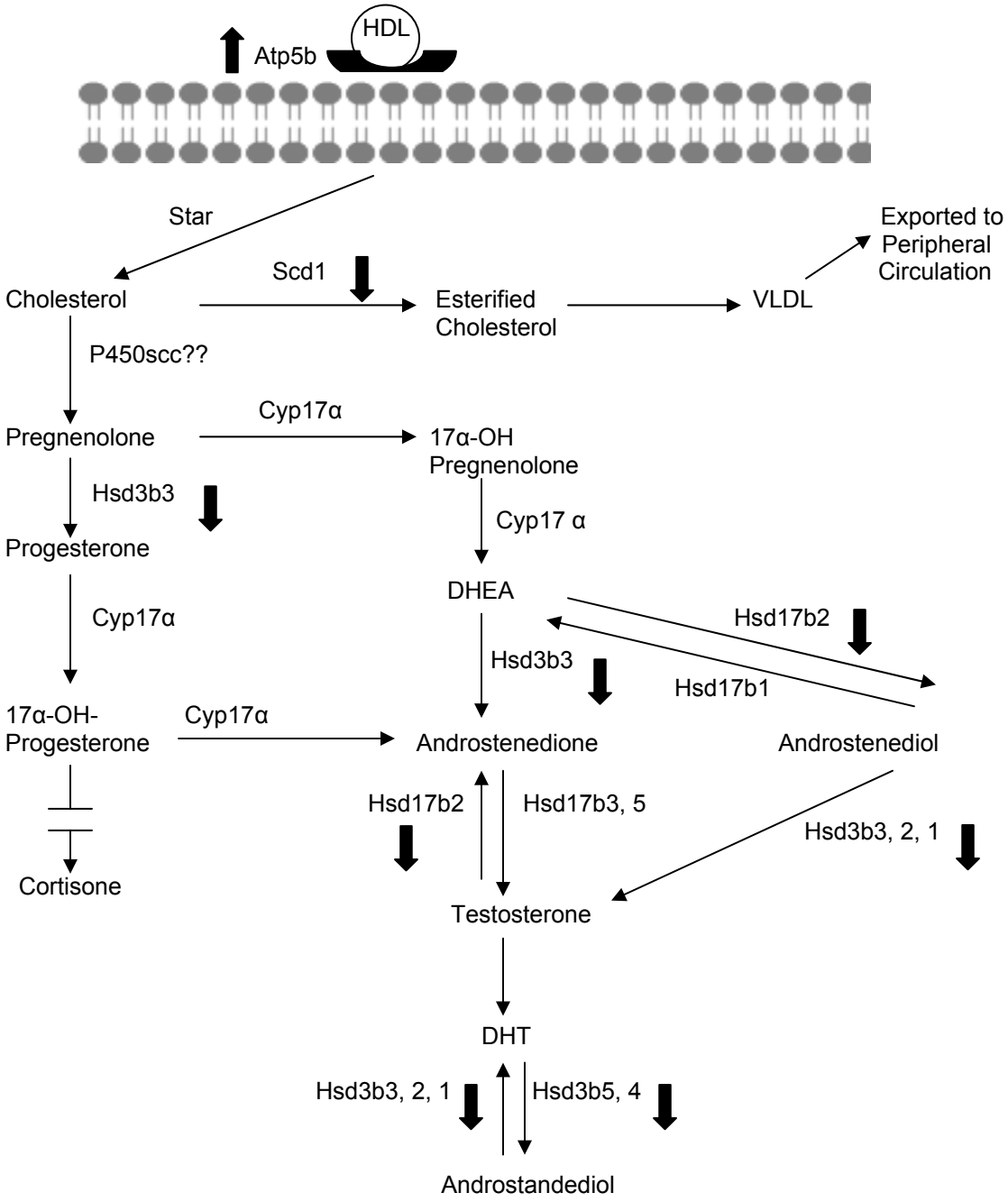

Figure 3: Flowchart showing gene expression changes in sex-steroid metabolism pathway in liver of mice fed the endophyte-infected (E+) diet. The bold arrows indicate up or down-regulated genes in response to the E+ diet, identified using microarray and qPCR. Symbol (??) indicate genes whose expression was not quantified in the present experiment. Gene names for the symbols used are defined in Table 3 and 4.

CHAPTER IV

TRANSCRIPTIONAL PROFILING OF MOUSE LIVER IN RESPONSE TO CHRONIC HEAT STRESS

Abstract

Heat stress adversely affects the health and well-being of both young and old animals. There is increased incidence of economic losses to livestock species due to prolonged summer heat stress conditions. Mice were used to study the effects of chronic heat stress on hepatic gene expression. Twenty-five mice were randomly allocated to either chronic heat stress (cHS; 34 ± 1°C) or thermoneutral (TN; 24 ± 1°C) conditions for a period of two weeks from 47 to 60 d of age. Liver genes differentially expressed due to cHS were identified using DNA microarrays. A two-stage ANOVA of 1353 genes queried using an oligoarray identified 30 genes differentially expressed between mice exposed to cHS or TN conditions. Hierarchical clustering of the 30 genes identified by ANOVA clearly separated the mice by treatment, with 100% confidence as computed by bootstrap analysis. Expressions of ten genes were also measured using quantitative real-time PCR. Genes involved in the anti-oxidant pathway and genes involved in metabolism were up-regulated due to cHS. Genes involved in generation of reactive oxygen radicals and a number of mitochondrial expressed genes were down-regulated by cHS. However, cHS did not produce an increase in oxidative stress induced mitochondrial DNA damage.

Introduction

Prolonged summer heat stress affects livestock by different physiologic mechanisms to reduce animal productivity. Across the United States, it is estimated that heat stress results in total estimated losses that are between $1.69 and $2.36 billion to livestock industries (St-Pierre et al., 2003). Chronic heat stress in rodents between an ambient temperature of 33-35°C has been shown to reduce growth, increase susceptibility to disease, and compromise reproduction in male and female rodents (Gordon, 1993).

In contrast, acute heat stress (aHS; animals exposed to 40-42°C for 24-48 h) has been studied extensively and shown to trigger a complex program of gene expression and biochemical adaptations (Zhang et al., 2002). The aHS is known to generate reactive oxygen radicals, oxidative damage, and alterations in intracellular signal transduction (Zhang et al., 2003). At the cellular level, it is generally accepted that aHS leads to increased expression of heat shock proteins (HSPs), and that the expression of these proteins is closely correlated with acquired thermotolerance (Horowitz, 2002). However, it is now clear that aHS involves more than expression of HSPs. A DNA microarray approach has been used to study the gene expression profile after an aHS in rats initially exposed to chronic heat (Horowitz et al., 2004). Genes involved in maintenance of DNA, cellular integrity, and cytoprotective signaling networks were identified as differentially expressed in the heart of rats.

Chronic mild heat stress (cHS) and acute intense heat stress (aHS) are really two very different challenges to animals. The latter results in hyperthermia and can lead to death if mice are exposed to intense HS for too long. However, mice are able to maintain normothermia at 34°C (Oufara et al., 1987). Nevertheless, the phenotypic changes that

were previously reported in response to cHS in various species (Gordon, 1993; St-Pierre et al., 2003) show that chronic mild HS alters animal performances and physiology. The molecular effects of chronic heat stress (cHS) have not been studied.

Thus, the objective of this study was to examine the transcriptional response to continuous chronic heat stress (cHS) in mice. Gene expression changes were studied in the liver as it has been previously shown that it is a prime target of tissue injury in physiological challenges such as aHS (Zhang et al., 2002).

Materials and Methods

Animals

Mice used in this study were of the outbred ICR strain (Harlan Sprague Dawley, Indianapolis, IN). In the present study, pups from 11 litters were weaned at 21 d of age. Twenty-five mice (13 males, 12 females) were randomly assigned to cHS conditions (34 ± 1°C; 12 mice) or thermoneutral (TN) conditions (24 ± 1°C; 13 mice) for a period of two wks from 47 to 60 d of age. Non-endophyte infected fescue seeds and rodent chow (Formulab Diet # 5008, PMI Feeds, St. Louis, MO) were ground to pass through a 1-mm screen and both components were mixed in equal parts.

All mice were housed in individual cages with relative humidity maintained at 35 to 50% and a 12:12 light-dark cycle with lights on at 0700. All the mice were weighed on d 1, 7, and 14 of the experiment. At the end of the experiment, mice were euthanized with carbon dioxide gas followed by cervical dislocation. Livers were obtained from all mice and were weighed, and tissues were frozen at -80°C. Data obtained were analyzed using

GLM procedure in SAS (SAS Inst. Inc., Cary, NC). The Animal Care and Use Committee, University of Missouri-Columbia, approved all procedures and protocols.

RNA extraction

Extraction of RNA was done using the RNAqueous-Midi kit (Ambion Inc., Austin, Texas) as described previously by Bhusari et al. (2006). The extracted RNA samples were kept in a -80ºC freezer until used.

Microarray preparation and hybridization

Microarrays were prepared by printing 1,353 oligos, 50-mers in length, representing rat genes expressed in liver, on Pan epoxy glass slides (MWG Biotech AG, Ebersberg, Germany). The microarray protocol including cDNA synthesis, hybridization and washing, scanning and data acquisition was previously described by Bhusari et al. (2006). The data generated from microarrays were stored in BioArray Software Environment database (BASE; Saal et al., 2002). The data on which this study is based have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the series accession number GSE2985.

Assessing technical variation due to microarray

We carried out 'self-self' hybridization for three different samples. Here, for each sample, equal amounts of RNA were labeled with Cy3, Cy5 fluorescent-dyes and co-hybridized on the same array. For all the genes on the array the mean ± 2SD was

calculated; this was 1.38 fold-change. Genes showing more than 1.38 fold-change were considered for differential gene expression.

Statistical analyses of microarray data

Microarray results were filtered in the BASE database to remove control spots, 3x Standard Saline Citrate (SSC), and blank spots. Raw intensities from each sample on a given array were used independently in an ANOVA model (Kerr and Churchill, 2001; Wolfinger et al., 2001). The log intensities for each array spot were statistically analyzed by using a mixed model approach consisting of two steps (Kerr and Churchill, 2001; Wolfinger et al., 2001). The first step involved array-specific normalization and the second step involved gene-specific analysis to test the effect of cHS vs TN on expression of individual genes. The normalization model in the first step was:

$$Y_{agp} = \mu + A_a + P_p + (AP)_{ap} + R_{agp} \qquad [1]$$

Where Y_{agp} represents the log base 2 of the observed fluorescent intensity signal from each gene on the array; μ is the overall mean value; A_a is the main effect of array a; P_p is the main effect of pin p (one of the 16 pins used to spot oligos on the array); $(AP)_{ap}$ is the effect of pin p by array a. The residual (R_{agp}) from the step 1 was obtained by subtracting the fitted values for the effects from the Y_{agp} values.

The second step of the statistical analysis consisted of fitting gene-specific models to the residuals (R_{agp}) obtained from the normalization approach discussed above. These models were:

$$R_{dgmt} = \mu_g + (GD)_{gd} + (GT)_{gt} + (GM_m(T))_{gmt} + \varepsilon_{dgmt} \quad [2]$$

Where μ_g is the mean value for gene *g*; $(GD)_{gd}$ is the gene *g* by dye *d* interaction; $(GT)_{gt}$ is the random interaction effect of gene *g* by treatment (cHS vs TN) *t* and these values were used to do gene-specific t-tests and to find differentially expressed genes between treatment groups; $(GM_m(T))_{gmt}$ is the effect of animal M_m within treatment *t* by gene *g*; ε_{dgmt} is the stochastic error obtained from the gene-specific model. These analyses were computed by using the Mixed Procedure in SAS (SAS Institute Inc., Cary, NC). The analyses were done using equal and unequal variances between treatment groups to obtain lists of differentially expressed genes. The probability values obtained from these analyses were used to find genes that had significantly different expression between treatment groups. Hierarchical clustering and support tree analyses were done using TIGR multiexperiment viewer software (TMEV; Saeed et al., 2003). The ontology of the genes shown in Tables 7 and 8 were obtained using DAVID (Dennis et al., 2003) and Locuslink from NCBI.

Quantitative real-time PCR

Genes for quantitative real-time PCR (qPCR) were chosen based on the gene lists obtained using ANOVA with equal and unequal variances and the *P*-values of genes. Expression profiles of 10 genes were measured with qPCR. From each mouse, 10 µg of total RNA were 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. Forward and reverse primer final concentrations were 100 nM in the SYBR green assay. Forward and reverse primer sequences are shown in Table 6. The reactions were performed using the Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). Primers were designed using Primer Express (Applied Biosystems, Foster City, CA) with an annealing temperature of 60°C and amplification size of less than 150 bp. The glyceraldehyde-3-phosphate dehydrogenase (Gapd) and β-actin cytoplasmic (β-actin) genes were each evaluated for use as an endogenous control by comparing their expression across four different cHS and TN samples at three different template concentrations per sample. β-actin was chosen as the endogenous control gene in our experiments as it had a lower coefficient of variation (CV, 0.03) across different samples and treatment groups compared to the Gapd (CV, 0.04). The qPCR was done in an ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification of gene expression changes were recorded after normalizing for β-actin 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 TN mice was used as a calibrator sample. Statistical analyses of the data were performed by comparing cHS C_T and TN C_T for each gene using a 2-tailed t-test with unequal group variance.

Oxidative DNA damage detection using XL-PCR

Genomic DNA samples were extracted from livers of cHS and TN mice and assayed using extra-long PCR (XL-PCR) to detect oxidative stress induced mitochondrial DNA (mtDNA) damage. The basis of this assay is that a DNA lesion will block DNA

polymerase and therefore lead to a decrease in amplification of the PCR product, which in this case is a 10 kb fragment of the mitochondrial genome (Santos et al., 2002). Thus, the decreased PCR product formation is indicative of mtDNA damage. A 10 kb fragment of mtDNA was amplified using the GeneAmp XL- PCR kit (Applied biosystems, Foster City, CA; Santos et al., 2002). Preliminary experiments indicated that PCR was kept in exponential phase using 15 cycles and 15 ng of DNA. The size of amplified product was verified using gel electrophoresis. Primer pair sequences used are listed in Table 6. The XL-PCR was initiated as a hot start at 75°C by addition of rTth enzyme, followed by initial denaturation at 94°C for 1 min, 15 cycles of 94°C for 15 sec, 65°C for 12 min. There was a final extension for 10 min at 72°C. All the reactions were performed in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). All DNA samples used for XL-PCR were amplified in duplicate and stored at -20°C until used.

SYBR green analysis of XL-PCR products. The XL-PCR product formed in the previous step was quantified using the SYBR green method of real-time PCR. Primers were designed in the middle of the 10 kb mtDNA (Table 6) to amplify a 120 bp fragment. It has been shown previously that the location of primers in the amplified XL-PCR product is not critical for quantification of amplified product obtained from the long-range PCR (Laws et al., 2001). Initial experiments were conducted to optimize the amount of template and primer concentrations so that PCR could be maintained in exponential phase. Subsequently, real-time PCR was performed using 5 μL of XL-PCR products diluted 1:1000 in water and 100 nM of primers. Real-time PCR product amplification was detected using the SYBR green method performed in an ABI 7500 sequence

detection system (Applied Biosystems, Foster City, CA) following standard default PCR conditions (user manual #2, ABI Prism 7700 SDS). For each sample, SYBR green detection of XL-PCR product was done in triplicate. Furthermore, a 117 bp fragment of mtDNA was amplified from 2.5 ng of DNA extracted from liver tissue to measure the mtDNA copy number in each sample. To calculate lesion frequency, the C_T obtained from XL-PCR product primers (X_s) were divided by the corresponding C_T obtained from mtDNA copy number primers (D_s) to account for potential copy number differences between samples. Normalized values from cHS ($X_s/D_s = A_d$) were compared with non-damaged controls (TN; $X_s/D_s = A_c$) resulting in relative amplification ratio (A_d/A_c). Assuming a random distribution of lesions and using the Poisson distribution ($f(x) = e^{\lambda}$ $\lambda^x/ x!$ where λ = the average lesion frequency) for the non-damaged template (zero class; $x = 0$), the average lesion frequency per strand was determined by using the following equation: $\lambda = -\ln A_d/A_c$ (Santos et al., 2002).

Measurement of anti-oxidant enzyme activities in HS mice liver

Anti-oxidant enzyme activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured in HS mice liver. The SOD activity was measured using an SOD assay kit (Superoxide Dismutase Assay kit, Cayman Chemical, Ann Arbor, MI). This assay uses a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine and absorbance is measured at 450 nm. This assay measures the activity of any type of SOD.

Catalase activity was measured based on its reaction with methanol in the presence of hydroperoxide (H_2O_2). The formaldehyde produced is measured

spectrophotometrically with purpald as chromogen, which gives a maximal absorbance at 540 nm (Catalase Assay kit, Cayman Chemical, Ann Arbor, MI). One unit is defined as the amount of enzyme that produces 1.0 nmol of formaldehyde per minute at 25 °C.

GPX activity was measured by a coupled reaction with glutathione reductase. The oxidized glutathione produced when GPX reduces H_2O_2 is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm, which is directly proportional to the GPX activity in the sample (GPX Assay Kit, Cayman Chemical, Ann Arbor, MI). This assay detects all of the GPX activities in the samples. One unit is defined as the amount of enzyme that oxidizes 1.0 nmol of NADPH to NADP+ per minute at 25°C. Protein concentrations were determined by the Bradford assay (Sigma Chemicals, St. Louis, MO).

Reduced glutathione (GSH) over oxidized glutathione (GSSG) ratio (GSH/GSSG) which is an indicator of oxidative stress was measured using BIOXYTECH GSH/GSSG-412 kit as per protocol (Oxis Research, Portland, OR).

Results

Phenotypic results

Mice exposed to cHS had reduced liver weights corrected for bodyweight compared to TN mice (1.26 ± 0.03 vs. 1.32 ± 0.03 g, for cHS and TN, respectively; P = 0.01).

Microarray results

Thirty differentially expressed genes were identified using ANOVA. The cHS resulted in the up-regulation of 18 genes, while 12 genes were down-regulated. Lists of up-regulated and down-regulated genes ($P \leq 0.05$) are in Tables 7 and 8, respectively. These genes clustered the mice into two groups that precisely reflect the cHS or TN treatments with 100% support from the bootstrap test. The genes were also separated into two groups of up-regulated or down-regulated genes with 100% support using hierarchical clustering (Saeed et al., 2003; Figure 4). Changes in liver gene expression in several important pathways were identified in response to cHS. Changes in expression of genes involved in ROS and anti-oxidant response (9 genes), carbohydrate and lipid metabolism (8 genes), cell proliferation (2 genes) and apoptosis (2 genes) were observed.

Quantitative real-time PCR results

Expressions of eight genes were verified using quantitative real-time PCR (qPCR) in liver samples from 11 cHS and 12 TN mice and these mice include the six cHS and seven TN mice used in the microarray experiment. The qPCR results of six genes were in agreement with microarray results in terms of direction of change and magnitude (Figure 5). All of these six genes (Aldh2, Psmb5, Scd1, Mt1, Csnk2a1, and Cyp2e1) had concordance in terms of statistical significance obtained using a two-way Student's t-test ($P < 0.05$). Protein kinase cAMP (Prkaa2) and catalase (Cat) qPCR results showed opposite direction fold change compared to that obtained with the microarray experiment (Prkaa2, up-regulation in cHS mice with microarray = 54%, down-regulation in cHS

mice with qPCR = 26%; Cat, down-regulation in cHS mice with microarray = 16%, up-regulation in cHS mice with qPCR = 32%), but differences were detected between treatment groups only for catalase ($P = 0.24$ and $P = 0.01$ for Prkaa2 and Cat, respectively). Expressions of two additional genes for which probes were not present on the arrays were analyzed using qPCR. The expression of glutathione peroxidase1 (Gpx1), an important anti-oxidant gene, was down-regulated in cHS mice by 29% compared to TN mice ($P = 0.05$). Expression of the superoxide dismutase 1 (Sod1) gene was up-regulated in cHS mice by 20% ($P = 0.04$). The highest fold change was observed for the Mt-1 gene, which was up-regulated by 136% in cHS mice.

Oxidative damage detection using XL-PCR

Previous reports indicated that acute HS (aHS) significantly increased oxidative stress and resulting ROS induced mtDNA damage (Zhang et al., 2003). Our gene expression data showed an increase in the expression of genes involved in the anti-oxidant response in mice under cHS. Therefore, XL-PCR was used to assess mtDNA damage. In cHS mice, there was 0.01 lesion/10 kb detected when compared to the control TN mice and the mtDNA damage detected was not different between treatment groups ($P = 0.57$). Thus, the cHS used in this study did not result in biologically relevant mtDNA damage.

Anti-oxidant enzyme activities

Catalase enzyme activity was higher in HS mice liver than the TN mice (36.7 ± 6.5 vs 16.2 ± 1.6, $P < 0.05$ for HS and TN mice respectively; Figure 7). The SOD enzyme

activity was higher in mice exposed to HS than the TN mice (27.8 ± 1.8 vs 19.5 ± 1.2, $P < 0.05$ for HS and TN mice respectively; Figure 7). Interestingly, the GPX enzyme activity was lower in HS mice liver compared to mice at TN (83.2 ± 10.7 vs 164.02 ± 12. 23, $P < 0.05$, for HS and TN mice respectively; Figure 8). Also, the GSH to GSSG ratio which is an indicator of oxidative stress was lower in HS mice liver compared to mice at TN (17.8 ± 1.2 vs 24 ±1.7, $P < 0.05$, for HS and TN mice respectively; Figure 8).

Discussion

Chronic HS (34°C for 2 wks) resulted in hepatic gene expression changes for genes involved in anti-oxidant response and metabolism. Liver weights were decreased by exposure to chronic mild heat stress compared to mice at TN. These differences between the two treatment groups show that cHS does indeed have an effect on the animals. Earlier studies have indicated that aHS resulted in differential expression of genes involved in anti-oxidant response, DNA repair, apoptosis, and metabolism in hepatic tissue (Zhang et al., 2002).

Anti-oxidant and stress gene expression

Chronic heat stress up-regulated a battery of anti-oxidant genes in mouse liver. Metallothionein-1 (Mt-1) was up-regulated by 136% as measured by microarray in cHS mice and this gene is involved in wide range of activities from heavy metal detoxification to ROS scavenging (Sato and Kondoh, 2002). In many in-vitro experiments, Mt-1 has been shown to be a potent scavenger of hydroxyl radicals, protecting DNA from oxidative damage and Mt-1 also has anti-apoptotic functions (Kang, 1999). The promoter

region of Mt-1 has an anti-oxidant response element (ARE) and thus expression of Mt-1 is increased in response to oxidative stress (Sato and Kondoh, 2002). Furthermore, nuclear localization of Mt-1 is prerequisite for protection of cell against oxidative stress, DNA damage, and apoptosis-mediated cell death (Levadoux-Martin et al., 2001). There are also indications that Mt-1 regulates activation of nuclear factor-κβ by tumor necrosis factor-α and nitric oxide signal transmission (Sato and Kondoh, 2002). Thus, up-regulation of Mt-1 in the present experiments could be in response to increased oxidative stress in mice exposed to cHS.

Chronic HS up-regulated the superoxide dismutase 1 (Cu/Zn Sod1) gene by 20% over the TN level as measured by qPCR. Superoxide dismutase (SOD) converts O_2^- into hydrogen peroxide (H_2O_2) that then produces a highly reactive hydroxyl radical (·OH) in the presence of reduced metal atoms, unless H_2O_2 is removed by the action of glutathione peroxidase or catalase (Figure 6). This is an important anti-oxidant enzyme since its point of attack is O_2^-, which is the initiator of the oxygen radical cascade that feeds into the lipid peroxidation chain reaction (Ahmad, 1995). Early work showed that Cu/Zn superoxide dismutase (Sod1) is predominantly found in the cytosol, with a small amount found in inner membrane space of mitochondria, whereas Mn superoxide dismutase (Sod2) is found in mitochondrial matrix (Brown et al., 2004). Acute HS at 42°C for 15 min in Sod1 knock-out mice resulted in an increase in ROS and oxidative stress, and caused accelerated impairment of spermatogonic cells. Up-regulation of Sod1 in the present experiment could be as a result of increased superoxide production due to cHS and its subsequent conversion into H_2O_2 by Sod1.

The catalase gene was up-regulated by cHS by 32% (qPCR) and is also involved in anti-oxidant response. Catalase acts sequentially to Sod1 to dismutase two molecules of H_2O_2 into water (Figure 6). Catalase has a relatively minor role in catabolism at lower levels of H_2O_2. However, the role of catalase is increased and becomes indispensable when H_2O_2 generation is enhanced by oxidative stress (Ahmad, 1995). Catalase gene expression was induced by 2-fold in young and old rats exposed to aHS at 38°C and its activity peaked 24 to 48 h after aHS (Zhang et al., 2002). In a recent study, transgenic mice overexpressing the human catalase gene had reduced oxidative damage, mitochondrial DNA mutations, and H_2O_2 generation, and had prolonged longevity compared to wild type mice (Schriner et al., 2005). Thus, catalase gene up-regulation in the present experiment indicates its protective action against cHS induced oxidative stress in mouse liver.

Interestingly, glutathione peroxidase (Gpx1) was down-regulated in cHS mice by 29% (qPCR) compared to TN mice. This gene is involved in the second step of oxidative metabolism, converting H_2O_2 to water (Figure 6). Thus, Gpx1 prevents conversion of H_2O_2 into the dangerous hydroxyl radical, and Gpx1 requires glutathione as reducing substrate during this reaction (Arthur, 2000). The Gpx1 overexpression and knockout models point to a role of this enzyme in protecting against oxidative attack of tissues (Arthur, 2000). In this study, we did not measure the expression of glutathione reductase which maintains a constant supply of glutathione from oxidized glutathione (Arthur, 2000). Glutathione is required for the activity of Gpx1 and reduced availability of glutathione might have resulted in reduced Gpx1 expression in cHS mice liver.

Expression of the mitochondrial aldehyde dehydrogenase gene (Aldh2) was up-regulated in cHS mice by 85% (array). This gene is mainly expressed in the mitochondrial matrix and plays a major role in the oxidation of acetaldehyde and protection of cells against oxidative stress (Ohsawa et al., 2003; Ohta et al., 2004). Oxidative stress and mitochondrial-derived ROS attack polyunsaturated fatty acid leading to membrane lipid peroxidation, thereby generating reactive aldehydes including 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde. A strong electrophile, 4-HNE has the ability to readily adduct cellular proteins and cause DNA damage (Ohsawa et al., 2003). It has been shown that Aldh2 deficient PC12 cells cannot rapidly metabolize 4-HNE and thus these cells are more susceptible to oxidative stress (Ohsawa et al., 2003). Therefore, Aldh2 up-regulation appears to be a response to counteract oxidative stress induced by cHS in mouse liver.

Expression of 'cytochrome P450, family 2, subfamily e, polypeptide 1' (Cyp2e1) was down-regulated in cHS mice by 42% (array). In liver microsomes, this enzyme is involved in an NADPH-dependent electron transport pathway and in xenobiotic metabolism. The Cyp2e1 gene product is able to produce oxidative stress directly through NADPH oxidase activity and through metabolism of xenobiotics (Gonzalez, 2005). The Cyp2e1-mediated oxidation of a variety of substrates is also believed to liberate a substantial amount of ROS that can lead to membrane lipid peroxidation and cell toxicity (Caro and Cederbaum, 2004). The activity of the Cyp2e1 gene promoter is inhibited by oxidative stress, suggesting a site for negative feedback control of ROS on Cyp2e1 gene expression (Caro and Cederbaum, 2004). The down-regulation of Cyp2e1 in cHS mice could be a part of a concerted adaptive response to oxidative stress, consisting not only of

repression of endogenous ROS-generating systems (ex: Cyp2e1), but also induction of anti-oxidant defenses like Cat, Aldh2, and Sod.

The proteasome (prosome, macropain) subunit, beta type 5 (Psmb5) gene was induced by 60% in cHS mice. It is a subunit of the catalytic 20S subunit of the 26S proteosome which is involved in degradation of oxidized proteins directly or by an ubiquitin-proteosomal pathway (Kwak et al., 2003). The promoter region of Psmb5 has tandem anti-oxidant response elements (ARE) and expression of this gene is increased in response to oxidative stress and is mediated by the binding of Nrf2 transcription factor to the ARE. Transcription factor Nrf2 is a central molecular target of indirect anti-oxidants, and products of the genes downstream of Nrf2 are a key mammalian defense system that enables adaptation to stresses and promotes cell survival (Kwak et al., 2003). The enhanced expression of Psmb5, a subunit of 20S catalytic core, due to cHS, might facilitate the removal of damaged proteins and provides an efficient means for cells to survive conditions of cHS.

Expression of casein kinase II, alpha 1 was increased by 70% in response to cHS (array). This serine/threonine kinase is involved in activation of Heat Shock Protein 1 during heat shock, and is required for activation of stress-induced kinase signaling (Soncin et al., 2003). This kinase has a direct role in the repair of chromosomal DNA strand breaks, maintenance of genetic integrity, and in proliferation and survival of mammalian cells by preventing caspase 8-induced apoptosis (Loizou et al., 2004).

The mitochondrial gene NADH dehydrogenase (ubiquinone) 1 alpha sub-complex 10 (Ndufa10) was upregulated by 20% in cHS mice. Expressions of the mitochondrial

genes, ATP synthase F0 subunit 8 (Atp8) and cytochrome oxidase 1, were down-regulated in cHS mice by 46% and 33%, respectively.

Overall changes in anti-oxidant and stress response gene expression (Csnk2a1, Psmb5) indicate that mice exposed to cHS might be experiencing oxidative stress and increased ROS production. To counteract the increased oxidative stress, there is up-regulation of genes involved in the anti-oxidant pathway (Cat, Sod1, Aldh2, and Mt-1) and down-regulation of the ROS generator like Cyp2e1.

Genes involved in metabolism

The ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit (Atp5b) gene was up-regulated by 50% in cHS mice (array). The beta subunit of mitochondrial ATP synthase (Atp5b) is encoded by a nuclear gene and assembled with the other subunits encoded by both mitochondrial and nuclear genes. The enzyme catalyzes ATP formation, using the energy of proton flux through the inner membrane during oxidative phosphorylation. The Atpb5 also acts as a high density lipoprotein (HDL) receptor for apolipoprotein-I on the cell surface of hepatocytes. Receptor stimulation by apoA-I triggers the endocytosis of HDL particles by a mechanism that depends strictly on the generation of ADP. Thus, this gene might be involved in HDL metabolism apart from its role in ATP biosynthesis in mitochondria (Martinez et al., 2003).

Chronic heat stress resulted in up-regulation of the ‘pyruvate kinase liver and RBC’ gene by 80% compared to mice at TN. This gene is involved in the final stage of glycolysis converting phosphoenolpyruvate into pyruvate. Stearoyl-CoA desaturase (Scd1) is a key rate-limiting microsomal enzyme involved in the biosynthesis of oleate

and palmitoleate and plays an important role in fat metabolism (Lee et al., 2004). This gene was up-regulated in response to cHS by 127% (qPCR). A previous report indicates that Scd1 deficiency increases basal thermogenesis through the β_3-adrenergic receptor-mediated pathway and increases lipolysis and fatty acid oxidation in brown adipose fat (Lee et al., 2004). Expression of hydroxyacid oxidase (glycolate oxidase) 3 was up-regulated in cHS mice by 50%. This peroxisomal enzyme is involved in fatty acid α-oxidation and utilizes the flavin mononucleotide cofactor to convert 2-hydroxy acids to 2-keto acids with the concomitant reduction of molecular oxygen to hydrogen peroxide (Jones et al., 2000). Expression of apolipoprotein B (ApoB) was down-regulated in cHS mice by 40% of that of TN mice. The gene product of ApoB is involved in cholesterol transport, and lipid and triacylglycerol mobilizations. It has been reported that oxidative stress and lipid peroxidation causes degradation of the ApoB protein (Pan et al., 2004). Expression of glutamine synthetase was down-regulated in cHS mice by 22%. This is a key enzyme in cellular nitrogen regulation; it facilitates the uptake of excitatory neurotransmitter glutamate, and is one of the enzyme that is rapidly induced by the stress hormone glucocorticoid. Decreased uptake of glutamate due to decreased glutamine synthetase activity could result in neurotoxic effects due to continued activation of N-methyl-D-aspartate receptor and generation of oxidants (Smith et al., 1991). Acute and chronic exercise in rats caused reduced glutamine synthetase activity in liver (Liu et al., 2000). This was hypothesized to be due to accumulation of oxidized glutamine synthetase protein and for the known vulnerability of glutamine synthetase to oxidation, as seen in aging studies (Smith et al., 1991).

Other differentially expressed genes due to cHS

Expression of cyclin-dependent kinase-4 (Cdk4) was up-regulated in cHS mice by 40%. This kinase belongs to the serine/threonine protein kinase family and is important for cell-cycle G1 phase progression and proliferation. The retinoblastoma binding protein 7 was down-regulated by cHS and is involved in chromatin remodeling, transcriptional repression, and has a role in cell proliferation and differentiation. Other genes such as plasminogen and sphingomyelin phosphodiesterase 1 were up-regulated by cHS and are involved in induction of apoptosis.

Anti-oxidant enzyme activities and oxidative stress

Anti-oxidant enzymes catalase (CAT) and superoxide dismutase (SOD) activities were increased in cHS mice liver ($P < 0.05$). Previous work involving acute heat stress in rat at 42°C has also shown an increase in CAT, SOD enzyme activities (Zhang et al., 2003). Thus, this increased activity could be to remove ROS generated by cHS and to metabolize superoxide radicals generated into hydrogen peroxides. The GSH/GSSG ratio is used as an indicator of oxidative stress (Zhang et al., 2003). This ratio was reduced in cHS mice liver, which indicates that mild long term HS is enough to produce increased oxidative stress and its effects. Also, GPX enzyme activity was reduced in cHS mice. This is an important anti-oxidant enzyme involved in similar reaction as catalase (conversion of hydrogen peroxide into water) but it has a higher K_m than catalase

(Forgione et al., 2002). Thus, a deficiency of GPX would theoretically lead to reduced elimination of ROS. The decreased GSH/GSSG ratio in cHS mice could be due to the reduced GSH levels. The reduced levels of GSH in cHS mice liver could have resulted in reduced GPX activity as GSH acts as a co-substrate for GPX enzyme activity.

Overall, the present experiments indicate that cHS leads to increased oxidative stress as indicated by reduced GSH/GSSG ratio and reduced GPX enzyme activity in HS mouse liver. This increased oxidative stress is being counteracted by increased activities of the anti-oxidant enzymes catalase and superoxide dismutase.

DNA damage and oxidative status in cHS mice

Acute heat stress at 42°C in rats has been shown to cause nuclear DNA damage in both young and old heat stressed rats (Zhang et al., 2003). In the present study, we were interested to see if cHS at 34°C for 2 wks would produce oxidative stress-induced mtDNA damage in mouse liver. We used the XL-PCR based approach followed by real-time PCR to quantify mtDNA damage (Santos et al., 2002). There was no biologically relevant mtDNA damage present in cHS mouse liver compared to the control mice kept at 24°C. In a previous study, old rats exposed to aHS had sustained increases in ROS and markers of oxidative damage which contributed to cellular damage and liver injury (Zhang et al., 2003). Lack of significant DNA damage observed in the present study could be due to an efficient DNA repair mechanism present in the young mice. It is also possible that the significant up-regulation of anti-oxidant enzymes seen in the present study could effectively scavenge the ROS and reduce the oxidative stress, thus reducing mtDNA damage. Thus, the cHS enhances the anti-oxidative networks thereby resulting in

a new physiological state of greater anti-oxidant/oxidant ratio suggesting an adaptive response. The lack of mtDNA damage observed in the present study fits with this interpretation of adaptive response in cHS mice.

Conclusions

The changes in gene expression and in liver weights observed in our experiment confirm that there is a cellular response to cHS. It is interesting that we do not detect statistically significant changes in HSPs gene expression levels. This is an indication that cHS might induce a somewhat different response than aHS. However, it is impossible to stress mice at 44^0C for two weeks, so the differences in HSPs expression levels might also be a consequence of the different end points used in previously reported aHS (a few hours to two days) and in our cHS challenge (two weeks). Interestingly, (Schwimmer et al., 2006) recently study gene expression in hypothalamus of rats raised at 34 ^{0}C for one month. They did not detect any significant changes in HSPs expression levels.

Implications

Oligonucleotide microarray and real-time PCR were used to identify genes differentially expressed in mouse liver in response to chronic heat stress. Many genes involved in the anti-oxidant pathway were up-regulated in chronic heat stressed mice. There was no mitochondrial DNA damage detected due to heat-stress induced oxidative-stress in mouse liver. Further analysis of oxidative stress biomarkers would be required to know the oxidation status of chronic heat stressed mice.

Table 6. Primer sequences (5' → 3') used in real-time PCR (gene accession numbers are listed in Tables 7 and 8)

Name	Symbol	Forward primer	Reverse primer	Product size
Actin, Beta Cytoplasmic	β-actin	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACAGAGT	75bp
Metallothienein-1	Mt-1	GCTGTGCCTGATGTGACGAA	AGGAAGACGCTGGGTTGGT	70bp
Catalase	Cat	CAGGCTCTTCTGGACAAGTACAAC	GCCGGCCTGCTGGTAG	69bp
Casein kinase II, alpha 1 polypeptide	Csnk2a1	GACCCCGAGAGTACTGGGATT	TGGCTTCAAACACTTCACTGTATTT	114bp
Aldehyde dehydrogenase 2, mito	Aldh2	GCCAATTACCTGTCCCAAGCT	GACTGGGCCCCAAACACAT	74bp
Protein kinase, AMP-activated	Prkaa2	TTCTGGACTTCAAAAGCATCGA	GAGGCCCGCAGCAGAAC	89bp
Superoxide dismutase 1(cu/zn)	Sod1	GGACCTCATTTTAATCCTCACTCTAAG	TGCCCAGGTCTCCAACATG	76bp
Glutathione peroxidase1	Gpx1	GACTGGTGGTGCTCGGTTTC	TGAGGGAATTCAGAATCTCTTCATT	81bp
Cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2E1	GATATTCCCAAGTCTTTAACCAAGTTG	CCACGATGCGCCTCTGA	82bp
Proteasome (prosome) subunit, beta type 5	Psmb5	CGCGAATCGAAATGCTTCA	TGTGGCCCGGGAATCC	86bp
Stearoyl-Coenzyme A desaturase 1	Scd1	TTC TTG CGA TAC ACT CTG GTG C	CGG GAT TGA ATG TTC TTG TCG T	98bp
10 kb mitochondrial DNA fragment	Mito 10	GCCAGCCTGACCCATAGCCATAATAT	GAGAGATTTTATGGGTGTAATGCCG	10kb
DNA copy number primer	DNC	CCCAGCTACTACCATCATTCAAGT	GATGGTTTGGGAGATTGGTTGATGT	117bp
SYBR primers for XL-PCR product detection	$SYBR_{XL}$	AAAAGCTCACTTGCCCACTT	TGTAAGCCGGACTGCTAATG	120bp

[a] Product size, bp includes the sequences of forward and reverse primers.

Table 7. List of genes up-regulated in liver due to chronic heat stress

Gene ID [a]	Symbol	Name	Gene ontology [b]	Ratio [c] (cHS/TN)	*P*-value
18870	PKLR	Pyruvate kinase liver	Isozyme of pyruvate kinase; glycolysis.	1.87	0.004
16456	F11r	F11 receptor	Protein binding, cell adhesion, integral to membrane.	1.49	0.008
67273	Ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	Oxidoreductase activity, nucleic acid metabolism.	1.18	0.01
11669	Aldh2	Aldehyde dehydrogenase 2, mito	Oxidoreductase activity, metabolism, mitochondria.	1.85	0.01
20597	Smpd1	Sphingomyelin phosphodiesterase 1, acid lysosomal	Sphingomyelin phosphodiesterase activity, carbohydrate metabolism.	1.19	0.01
56307	Metap2	Methionine aminopeptidase 2	Proteolysis and peptidolysis.	1.25	0.02
108124	Napa	N-ethylmaleimide sensitive fusion protein	Intracellular transporter activity, protein transport, golgi.	1.36	0.02
17748	Mt1	Metallothionein 1	Metal ion homeostasis, cytosol	2.36	0.02
19173	Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	Peptidase activity, ubiquitin dependent protein catabolism, cytosol.	1.56	0.02
26378	Decr2	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal	Oxidoreductase activity, metabolism, peroxisome.	1.28	0.02
11947	Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	Nucleotide binding, ATP synthesis, mitochondria.	1.48	0.03
18815	Plg	Plasminogen	Hydrolase activity, induction of apoptosis.	1.29	0.04
12567	Cdk4	Cyclin-dependent kinase 4	Protein kinase activity, regulation of Cell cycle.	1.41	0.04
56185	Hao3	Hydroxyacid oxidase (glycolate oxidase) 3	Oxidoreductase activity, protein targeting.	1.47	0.04
72433	Rab38	Rab38, member of RAS oncogene family	Protein kinase, transporter activity.	1.46	0.05
20249	Scd1	Stearoyl-Coenzyme A desaturase 1	Oxidoreductase, lipid biosynthesis activity.	2.25	0.05
78975	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	Protein kinase activity, response to stress.	1.55	0.05
12995	Csnk2a1	Casein kinase II, alpha 1 polypeptide	Protein kinase activity, protein kinase CK2 complex component.	1.69	0.05

[a] Available: http://www.ncbi.nlm.nih.gov/entrez.
[b] Ontology classification of genes according to biological process, molecular function and cell location obtained using DAVID (http://david.niaid.nih.gov/david/).
[c] Ratio (cHS/TN) for up-regulated genes computed from the expression of the gene in mice exposed to chronic heat stress (cHS) over its expression in mice at thermoneutrality (TN).

Table 8. List of genes down-regulated in liver due to chronic heat stress

Gene [a] ID	Symbol	Name	Gene ontology [b]	Ratio [c] (cHS/TN)	*P*-value
17706	ATP8	ATP synthase F0 subunit 8	Hydrogen-translocating F-type ATPase complex, mitochondrial membrane.	0.55	0.002
15108	Hadh2	Hydroxyacyl-Coenzyme A dehydrogenase type II	Oxidoreductase activity, metabolism, mitochondria.	0.68	0.01
54140	Avpr1a	Arginine vasopressin receptor 1A	Vasopressin receptor activity, signal transduction, membrane.	0.73	0.02
245688	Rbbp7	Retinoblastoma binding protein 7	Transcriptional repressor, chromatin remodeling.	0.81	0.03
15925	Ide	Insulin degrading enzyme	Proteolysis and peptidolysis.	0.93	0.03
18670	Abcb4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	ATPase activity, transporter activity.	0.75	0.03
14645	Glul	Glutamine synthase	Ligase activity, glutamine biosynthesis, mitochondrion.	0.78	0.04
21788	Tfpi	Tissue factor pathway inhibitor	Protease inhibitor activity, blood coagulation.	0.67	0.05
12359	Cat	Catalase	Oxidoreductase activity, response to oxidative stress.	0.84	0.04
238055	Apob	Apolipoprotein B	Lipid, triacylglycerol metabolism.	0.61	0.04
	J01435	mitochondrial cytochrome oxidase subunits I,II, III genes		0.67	0.04
13106	Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	Oxidoreductase, monooxygenase activity, electron transport.	0.58	0.04

[a] Available: http://www.ncbi.nlm.nih.gov/entrez.

[b] Ontology classification of genes according to biological process, molecular function and cell location obtained using DAVID (http://david.niaid.nih.gov/david/).

[c] Ratio (cHS/TN) for down-regulated genes computed from the expression of the gene in mice exposed to chronic heat stress (cHS) over its expression in mice at thermoneutrality (TN).

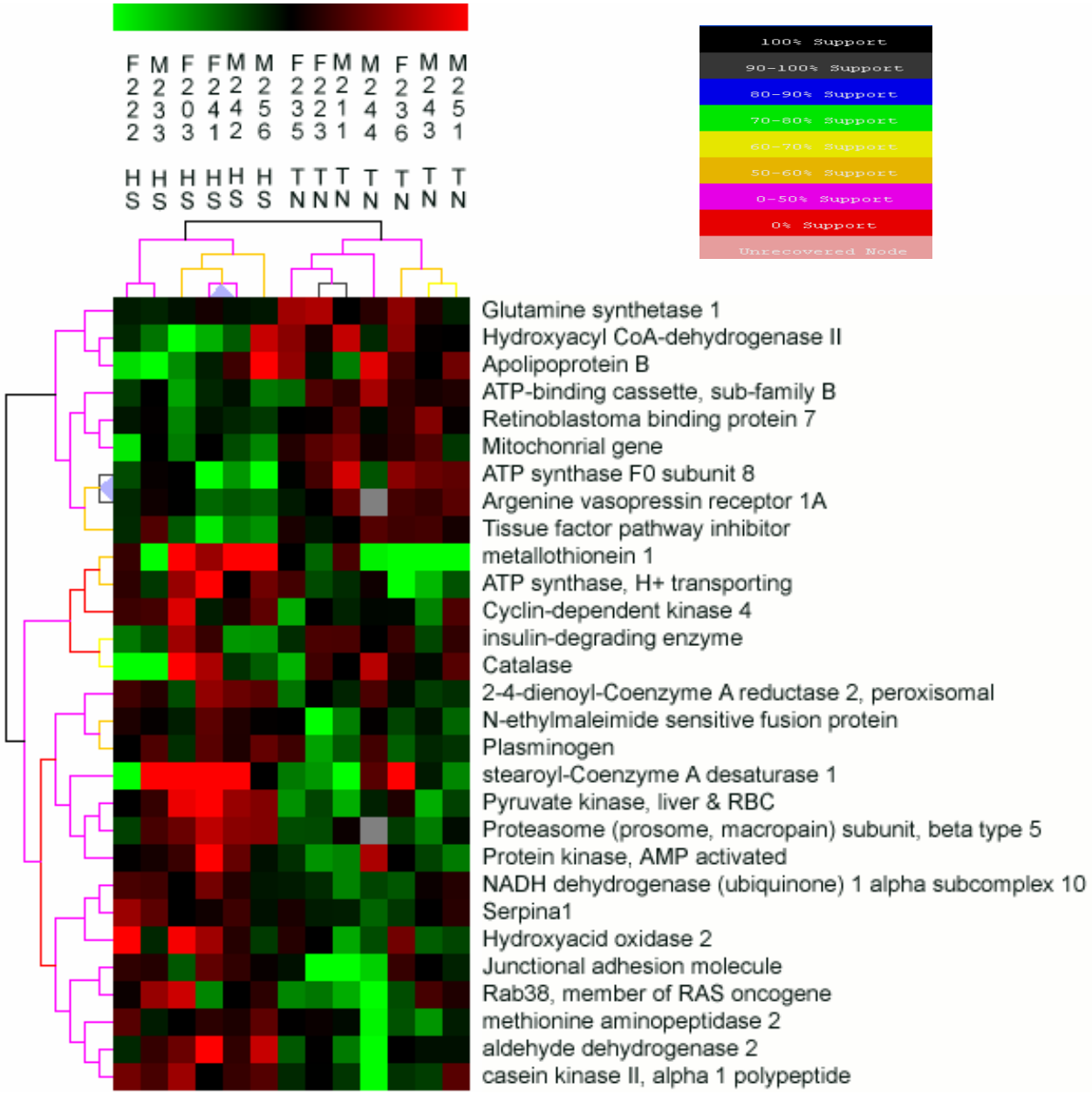

Figure 4: Differentially expressed genes ($P \leq 0.05$) and mice clustered using TIGR multi-experiment viewer. The bootstrap analysis was done with 400 iterations to assess the significance of the hierarchical trees. The mice are divided into two groups with 100% support based on their treatment (top clustering) and 18 genes were up-regulated while 12 genes were down-regulated (clustering shown at right) by chronic heat stress (cHS). The green blocks represent the down-regulated genes, the red blocks represent up-regulated genes, and the black blocks represent genes whose expression is not changed due to cHS. Symbol 'F' indicate female and 'M' indicate male along with the mice numbers at the top of the diagram.

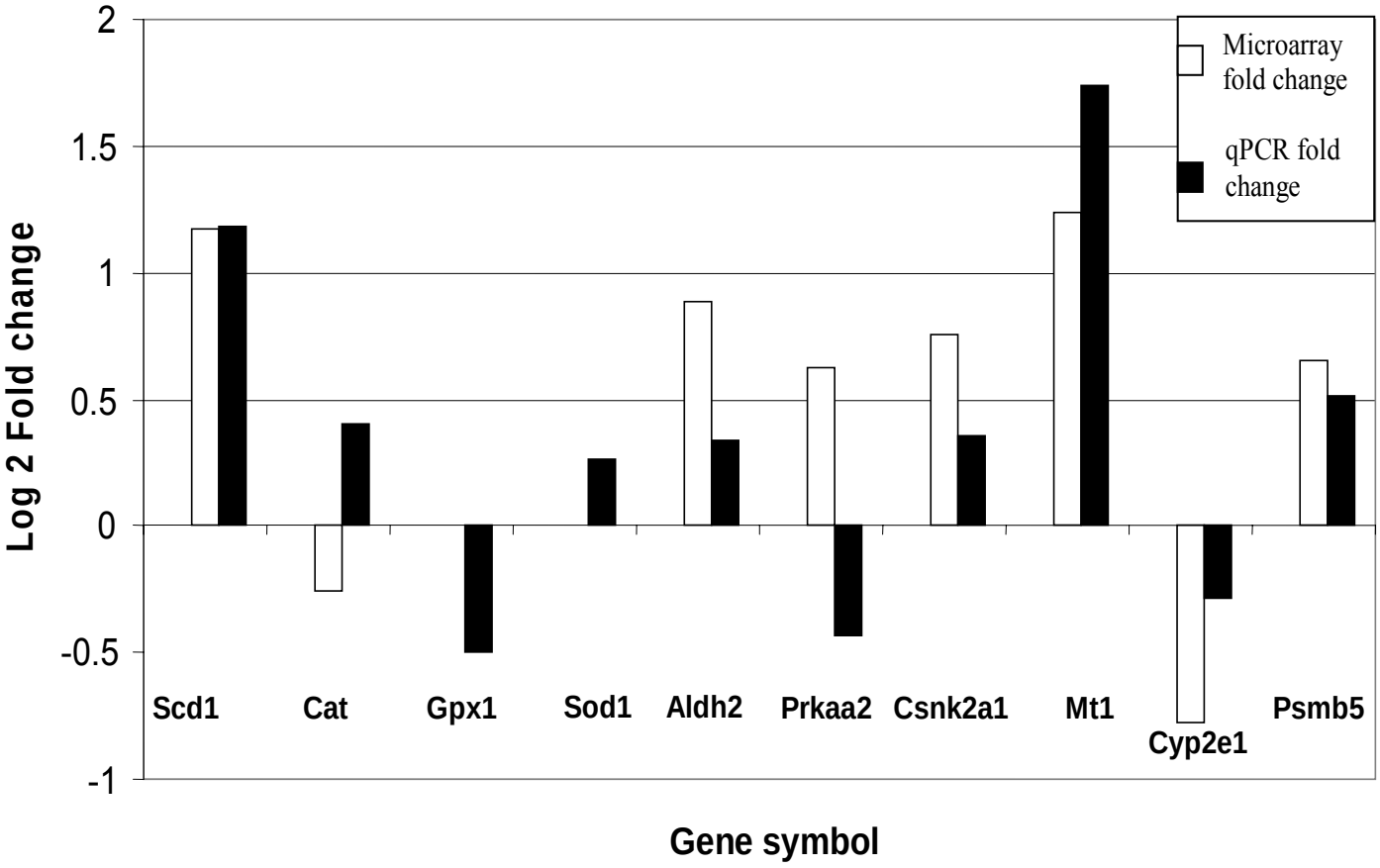

Figure 5: Expression analyses of 10 genes using oligonucleotide microarrays and quantitative PCR (qPCR). Showing fold-change computed from the expression of genes in mice exposed to chronic heat stress (cHS) over its expression in mice exposed to control thermoneutral (TN) temperature. Open and solid bars represent microarray or qPCR data, respectively. Additional information about gene symbols can be obtained from www.ncbi.nlm.nih.gov/entrez.

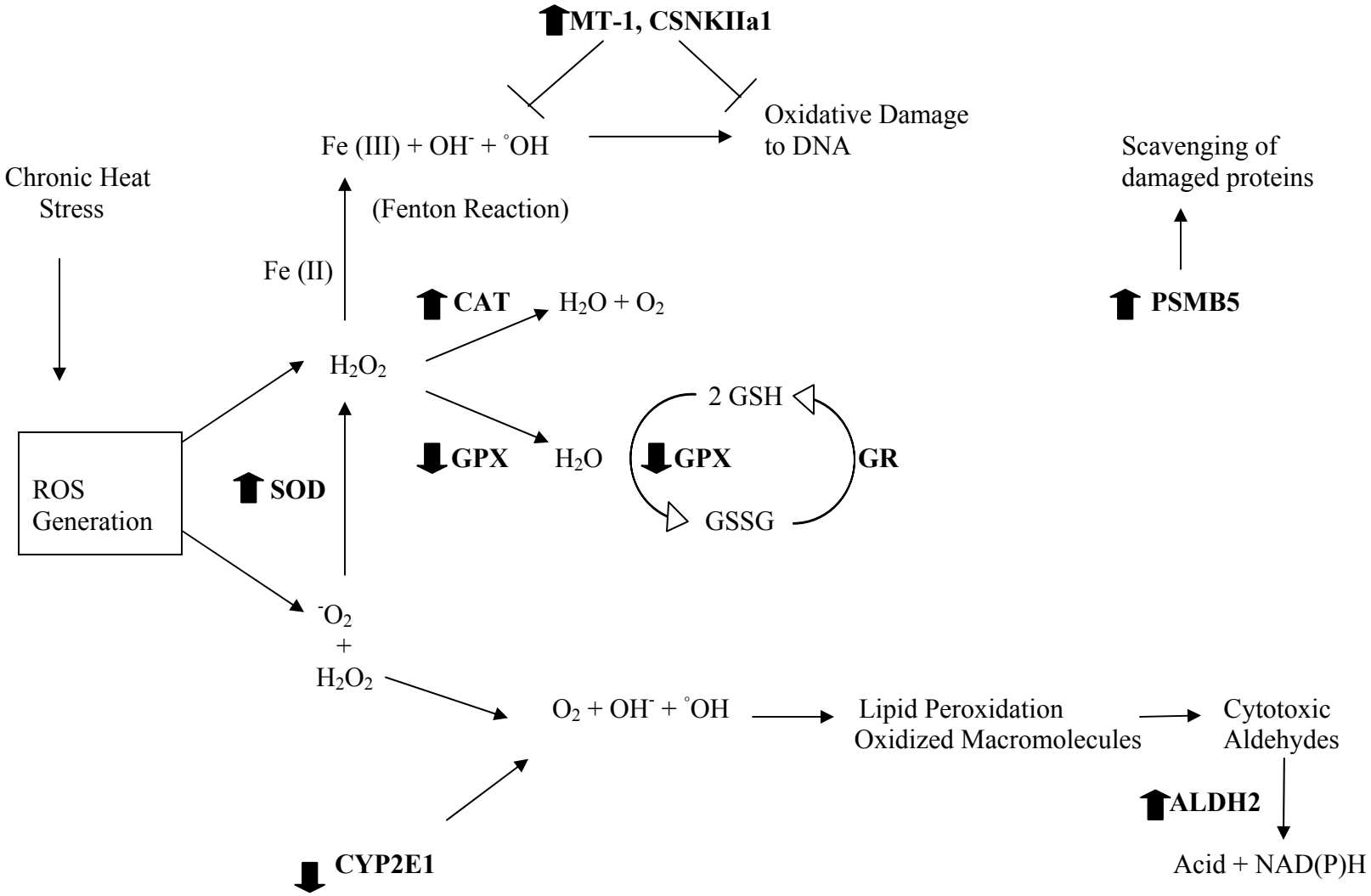

Figure 6: Flowchart showing gene expression changes in the anti-oxidant pathway in liver of mice exposed to chronic heat stress (cHS). The bold arrows indicate up or down-regulated genes in response to the cHS identified using microarray and qPCR. Gene names for the symbols used are defined in Tables 7 and 8.

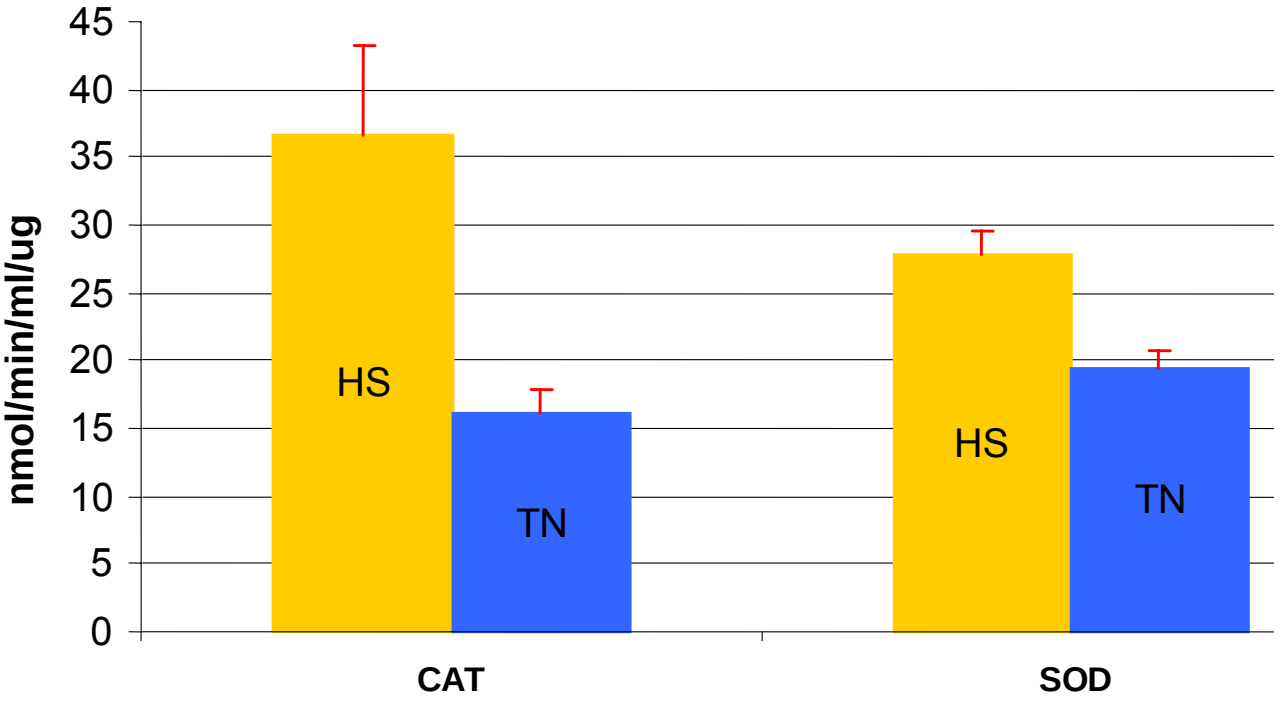

Figure 7: Catalase (CAT) and Superoxide Dismutase (SOD) enzyme activities in heat stress (HS), and thermoneutral (TN) mouse liver.

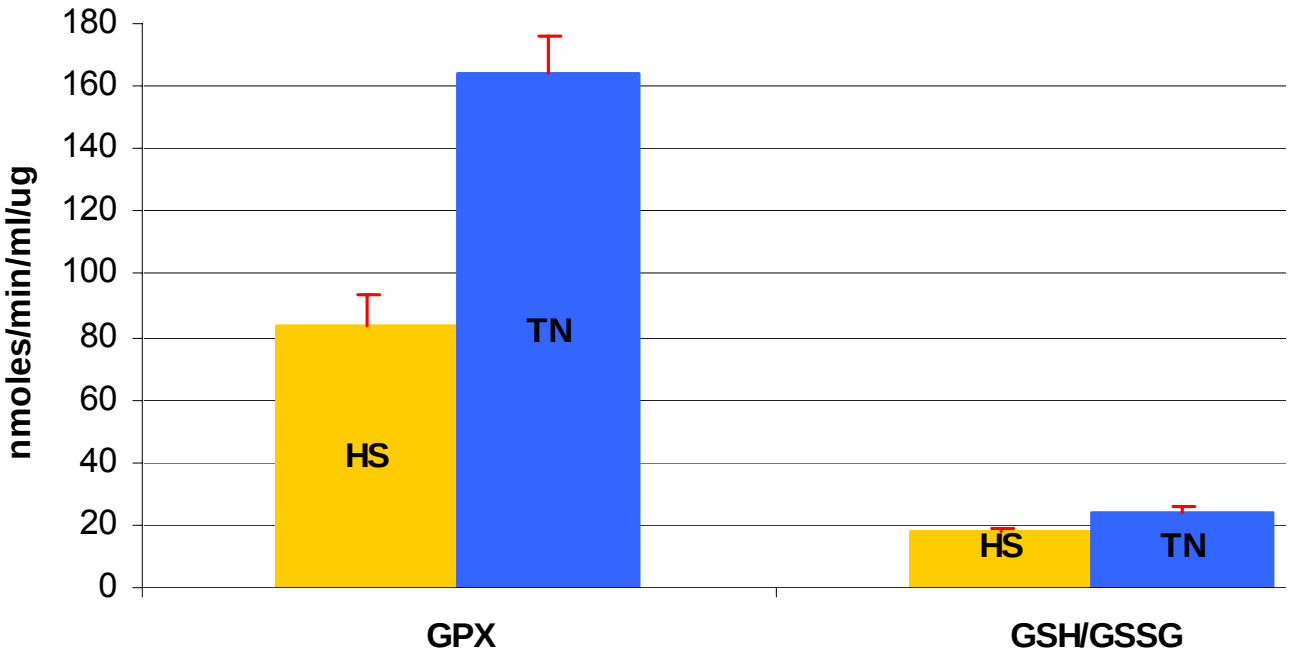

Figure 8: Glutathione peroxidase (GPX) enzyme activity, reduced glutathione to oxidized glutathione ratio in heat stress (HS), and thermoneutral (TN) mouse liver.

CHAPTER V

EFFECTS OF HEAT STRESS ON ALTERATIONS IN MURINE HEPATIC GENE EXPRESSION ASSOCIATED WITH FESCUE TOXICOSIS.

Abstract

Fescue toxicosis affects wild and domestic animals consuming ergot alkaloids contained in tall fescue forage infected with the endophytic fungus, *Neotyphodium coenophialum*. When animals are consuming infected fescue forage during periods of elevated ambient temperatures (summer), a range of phenotypic disorders collectively called summer slump is observed. It is characterized by hyperthermia, with an accompanying decrease in feed intake, growth, milk yield and reproductive fitness. Laboratory mice also exhibit symptoms of fescue toxicosis at thermoneutral temperature, as indicated by reduced growth rate and reproductive fitness. Our goal was to characterize the differences in gene expression in liver of mice exposed to summer-type heat stress (HS) and infected fescue (E+) when compared to mice fed infected fescue at thermoneutral temperature. Mice were fed E+ diet under HS (34 ± 1°C; n = 13; E+HS) or thermoneutral (TN) conditions (24 ± 1°C; n = 14; E+TN) for a period of two weeks between 47 to 60 d of age. Genes differentially expressed between E+HS versus E+TN were identified using DNA microarrays. Forty-one genes were differentially expressed between treatment groups. Expressions of eight genes were measured using quantitative real-time PCR.

Genes coding for phase I detoxification enzymes were up-regulated in E+HS mouse liver. This detoxification pathway is known to produce reactive oxidative species. We observed an up-regulation of genes involved in the protection against reactive oxidative species.

Key genes involved in *de novo* lipogenesis and lipid transport were also up-regulated. Finally, genes involved in DNA damage control and unfolded protein responses were down-regulated.

Introduction

Intake of ergot alkaloids found in tall fescue grass infected with an endophyte, *Neotyphodium coenophialum,* produces a detrimental condition known as fescue toxicosis. The endophytic fungus produces ergot alkaloids, mostly ergopeptine alkaloids such as ergovaline, which are responsible for fescue toxicosis**.** Signs of fescue toxicosis include decreased feed intake, weight gain, and milk production, and peripheral vasoconstriction, and rough hair coat (Schmidt and Osborn, 1993; Thompson and Stuedemann, 1993). Summer slump is a complex disease syndrome that occurs during heat stress periods, when animals are consuming infected fescue forage, and is characterized by hyperthermia, with an accompanying decrease in feed intake and growth. Hyperthermia and poor body weight gain from the toxicosis are most severe at the onset of high ambient temperature and increased relative humidity during the spring and summer (Hemken et al., 1981). Rectal temperature and respiration rate also increased in E+ fed heifers and steers under HS conditions, but not at cooler temperatures (Hemken et al., 1981). Laboratory mice were used previously as a model for fescue toxicosis because these animals exhibit reduced growth, reproduction, and lactation when fed an E+ diet (Miller et al., 1994; Zavos et al., 1987). Consumption of an E+ diet by mice at TN resulted in changes in expression of genes involved in carbohydrate, cholesterol, and lipid metabolism (Bhusari et al., 2006). The objective of this experiment was to identify

hepatic gene expression changes in mice fed an E+ diet under HS condition. In an attempt to understand the interaction between HS and E+ on hepatic gene expression in mouse liver, mice were exposed to TN temperatures or controlled HS while consuming E- or E+ diet. In mammals, the liver plays an important role in detoxification and metabolism of toxins, and is a prime target of tissue injury in response to various physiological challenges like HS. Consequently, we use microarray-based expression profiling to study the transcriptional response of mouse liver genes to the ergopeptine alkaloids and heat stress by comparing E+HS to E+TN mice.

Materials and Methods

Animals

Mice used in this study were of the outbred ICR strain (Harlan Sprague Dawley, Indianapolis, IN). Pups from 11 litters were weaned at 21 d of age. Thirteen male and 14 female mice were first fed an E- diet for one week then switched to a E+ diet under HS (34 ± 1°C; n = 13; E+HS) or TN conditions (24 ± 1°C; n = 14; E+TN) from 47 to 60 d of age. Endophyte-infected fescue seeds and rodent chow (Formulab Diet # 5008, PMI Feeds, St. Louis, MO) were ground to pass through a 1-mm screen and mixed in equal part. Composition of control and treatment diets were as previously described, and seed ergovaline (EV; the major ergot alkaloid associated with fescue toxicosis; Spiers et al., 2005) levels were measured. Control (E−) seed contained 22 ppb of EV, whereas E+ 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). Feed and water were provided *ad libitum* throughout the study. All mice were housed in individual cages with relative

humidity maintained at 35 to 50% and a 12:12 light-dark cycle with lights on at 0700. Mice were weighed on d 1, 7, and 14 of the experiment. At the end of the experiment, mice were euthanized with carbon dioxide gas followed by cervical dislocation. Livers were weighed and frozen at -80°C. The Animal Care and Use Committee, University of Missouri-Columbia, approved all procedures and protocols. Only mice fed the E+ diet under TN or HS conditions were used in the microarray and quantitative PCR experiments.

RNA extraction

Extraction of RNA was done using the RNAqueous-Midi kit (Ambion Inc., Austin, Texas) as described previously by Bhusari et al. (2006). The extracted RNA samples were kept in a -80ºC freezer until used.

Microarray preparation and hybridization

Microarrays were prepared by printing 1,353 oligos, 50-mers in length, representing rat genes expressed in liver, on Pan epoxy glass slides (MWG Biotech AG, Ebersberg, Germany). The microarray protocol, including cDNA synthesis, hybridization and washing, scanning and data acquisition, was described by Bhusari et al. (2006). Extracted RNA from each of the 6 (3 male, 3 female) randomly selected E+HS mice and 6 (3 male, 3 female) E+TN mice treatment groups were individually hybridized to the array with reference RNA (Universal Mouse Reference RNA, Stratagene, La Jolla, CA) in a reference microarray design. In a reference design, each experimental sample is hybridized against a common reference RNA sample (Churchill, 2002). Two or three

replicates (arrays) were done per animal, which are done in a dye swap design. The data generated from microarrays were stored in the BioArray Software Environment database (BASE; Saal et al., 2002).

Assessing technical variation due to microarray

We carried out 'self-self' hybridizations for three different samples. For each sample, equal amounts of RNA were labeled with Cy3 and Cy5 fluorescent-dyes and co-hybridized on the same array. The mean ± 2SD fold-change was calculated for all the genes on the array (1 ± 1.38). To remove false positives but avoid being too conservative, we decided that genes showing more than ± 1.30 fold-changes were considered for differential gene expression.

Statistical analyses of microarray data

Microarray results were filtered in the BASE database to remove control spots, 3x Standard Saline Citrate (SSC), and blank spots. Then the background-corrected-median-intensities were normalized using external plant control genes using GenePix Pro 4.0.1.12 software (Axon Instruments Inc, CA). The normalized intensities were then inputted into the software package MAANOVA (Wu et al., 2003) to model the data and run statistical analyses. Intensities were first log2 transformed and then a 2-stage ANOVA model was applied (Wolfinger et al., 2001). The first stage was the normalization model to remove the effect of array and dye at the across gene level

$$1 - \quad y_{gnlijk} = \mu + A_i + D_k + (AD)_{ik} + \varepsilon_{gnlijk}$$

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.

$$2 - \varepsilon_{gnlijk} = (GV)_{gi} + (GD)_{gk} + r_{gnlijk}$$

For these models $y_{gnlijk} = \log_2(w_{gnlijk})$, 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, ε and r are the residuals from the linear models.

A permutation Fs 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 Fs test *P*-value less than 0.05 were regarded as significantly differentially expressed across samples. The filtered list was then input into the TIGR multiexperiment viewer software (Saeed et al., 2003) to run hierarchical clustering. Gene ontology was obtained using 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. The microarray data files on which this paper is based have been deposited with National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with series number GSE5642.

Quantitative real-time PCR

Genes for quantitative real-time PCR (qPCR) were chosen based on the gene lists obtained using MAANOVA and the *P*-values of genes. Expression profiles of eight genes were measured with qPCR. From each mouse, 10 µg of total RNA were 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. Forward and reverse primer final concentrations were 100 nM in the SYBR green assay. Forward and reverse primer sequences are shown in Table 9. The reactions were performed using the Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). Primers were designed using Primer Express (Applied Biosystems, Foster City, CA) with an annealing temperature of 60°C and amplification size of less than 150 bp. β-actin was chosen as the endogenous control gene in our qPCR experiments. The qPCR was done in an ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification of gene expression changes were recorded after normalizing for β-actin 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 mice was used as a calibrator sample. Statistical analyses of the data were performed by comparing E+HSC_T with E+TNC_T for each gene using a 2-tailed t-test with unequal group variance.

Results

Microarray work

Randomly chosen six E+TN (out of 14) and six E+HS (out of 13) mice were used in a microarray experiment to detect gene expression changes. The analysis identified 41 genes ($P \leq 0.05$) as differentially expressed between treatment groups (E+TN versus E+HS). The E+HS resulted in up-regulation of 33 genes, while eight genes were down-regulated. Lists of differentially expressed genes ($P \leq 0.05$) are shown in Table 10. Principal component analysis was used to classify the mice based on the differentially expressed genes. One of the mice belonging to E+TN group clustered with E+HS mice

(Figure 9). Genes involved in reactive oxygen species (ROS) and anti-oxidant response (seven genes), lipid metabolism (14 genes), xenobiotic metabolism and detoxification (11 genes) were identified as differentially expressed in response to E+HS (Table 10).

Quantitative real-time PCR results

Expressions of eight genes were verified using qPCR in liver samples from 13 E+HS and 14 E+TN mice. These mice include the six E+HS and the six E+TN mice used in the microarray experiment. The qPCR results of seven genes were in agreement with microarray results in terms of magnitude and direction of change (Figure 10). Six of these seven genes (Lsr, Cyb3r5, Cyp3a25, ApoB, Acly, and Mt1) had concordance in terms of statistical significance using a two-way Student's t-test ($P < 0.05$). For Fmo5, qPCR results showed opposite direction fold change compared to that with the microarray experiment (Fmo5, down-regulation in E+HS mice with microarray = 40%, up-regulation in E+HS mice with qPCR = 42%, $P < 0.05$).

Discussion

Lipid and cholesterol metabolism

Numerous genes involved in lipid and cholesterol metabolism are differentially expressed in mice exposed to E+HS. ATP-citrate lyase (Acly) was up-regulated in E+HS mice by 228% (qPCR) compared to E+TN mice. This gene codes for a cytosolic enzyme that catalyzes the formation of acetyl-coenzyme A (CoA) and oxaloacetate from citrate and CoA. The acetyl-CoA formed is a key building block in *de novo* lipogenesis

(Beigneux et al., 2004). The gene product of Acly is required for the export of lipid building blocks from mitochondria and for lipid biosynthesis in cytosol.

The fatty acid synthase (Fasn) gene was up-regulated in E+HS mice by 48% compared to E+TN mice. The Fasn protein is a central enzyme in *de novo* lipogenesis in mammals and catalyzes all the reactions for the conversion of acetyl-coA and malonyl-CoA to palmitate. The products of Fasn, palmitate and stearate (C18), serve as substrates for chain elongation to produce very-long-chain fatty acids. These fatty acids are important constituents of sphingolipids, ceramides, and glycolipids that are needed for cell division progression and brain structures and neurological functions (Chirala et al., 2003).

Thyroid hormone responsive SPOT14 homolog (S14) was up-regulated in E+HS mice by 64%. The Spot 14 gene is mainly expressed in lipogenic tissues such as liver, adipose tissue and mammary gland and is associated with *de novo* lipogenesis (Zhu et al., 2005). The Spot 14 protein functions as a transcription factor necessary for the induction of lipogenic enzyme gene expression (Kinlaw et al., 1995).

Expression of the acyl-coA synthetase 1 (Acsl1) gene was up-regulated by 58% in E+HS mice (microarray). Acsl1 catalyses the ATP-dependent acylation of fatty acids into long chain acyl-coAs and is the first step in lipid metabolism following entry of fatty acid into the cell (Parkes et al., 2006). The Acsl1 gene product is linked to the storage pathway of lipid metabolism in liver and may act to channel fatty acids into triglyceride synthesis rather than into β-oxidation and energy release (Parkes et al., 2006).

The lipolysis-stimulated receptor (Lsr) gene was up-regulated in E+HS mice by 84% (qPCR) compared to E+TN mice. The Lsr gene product is a lipoprotein receptor

primarily expressed in the liver and steroidogenic tissues (Mesli et al., 2004). The protein binds lipoproteins containing apoB and apoE (Mesli et al., 2004). Lsr is considered to be involved in a rate-limiting step for the clearance of dietary triglycerides and plays a role in determining their partitioning of lipids between the liver and peripheral tissues (Yen et al., 1999).

Expression of Apolipoprotein H (ApoH), primarily expressed in liver was up-regulated in E+HS mice by 88%. About 40% of ApoH has been identified as a constituent of chylomicrons, VLDL and HDL. Apolipoprotein H is involved in lipoprotein metabolism (Polz and Kostner, 1979a, b).

Diazepam binding inhibitor (Dbi), also called acyl-CoA binding protein (Acbp), was up-regulated in E+HS mice by 32%. Acyl-CoA-binding protein is an intracellular lipid-binding protein that selectively binds medium and long chain acyl-CoA esters (C14–C22) (Sandberg et al., 2005). It plays an important role in intracellular acyl-CoA transport to the mitochondria and cellular pool formation. Disruption of the Acbp gene in yeast indicates its involvement in the synthesis of very long chain fatty acids and it is required for proper protein sorting and vesicular trafficking (Gaigg et al., 2001). Dbi/Acbp is also involved in cholesterol translocation across mitochondrial membranes, a rate-limiting step in the biochemical synthesis of steroids (Swinnen et al., 1998).

Overall, there is up-regulation of genes coding for rate-limiting enzymes involved in *de novo* lipogenesis in E+HS mice (Table 10). Genes involved in transport and partitioning of lipids such as lipolysis-stimulated receptor and acyl-CoA binding protein (Table 10) were also differentially expressed, which would increase transport of lipids into the liver for down-stream processes.

Detoxification gene expression changes

Expression of cytochrome b5 reductase 3 (Cyb5r3) was up-regulated in E+HS mice by 60% (Table 10, Figure 10). The Cyb5r protein mediates electron transfer from NADH to fatty acid desaturases, P450 oxidases (Hildebrandt and Estabrook, 1971), and plays a direct role in xenobiotic metabolism (Kurian et al., 2004). This gene is also involved in modulating activities of the Cyp17 gene, which is essential to steroidogenesis (Akhtar et al., 2005).

The flavin containing monooxygenase 5 (Fmo5) gene was up-regulated in E+HS mice by 42% (Figure 10, Table 10). Flavin-containing monooxygenases are microsomal enzymes that catalyze the NADPH and oxygen-dependent oxidation of many important drugs and xenobiotics (Ziegler, 2002).

Expression of epoxide hydrolase 2, cytoplasmic (Ephx2) was up-regulated in E+HS mice by 40%. Epoxides are a class of compounds that can arise from the cytochrome P450–mediated oxidation of alkenes, aromatic hydrocarbons, heterocycles. Epoxide hydrolases (EH) hydrolyze toxic epoxides into their corresponding diols which are typically less reactive, more water soluble, and more easily excreted from the body (DuTeaux et al., 2004). In a recent study, an E+ diet fed to rats resulted in an increased expression of the epoxide hydrolase 1 gene (Settivari et al., 2006).

Cytochrome P450, family 2, subfamily a, polypeptide 12 (Cyp2a12) and cytochrome P450, family 2, subfamily d, polypeptide 26 (Cyp2d26) genes were up-regulated in E+HS mice by 42 and 49%, respectively (Table 10). Cytochrome P450,

family 3, subfamily a, polypeptide 25 (Cyp3a25) was down-regulated in E+HS mice by 60 (Table 10).

Metabolism of xenobiotic compounds involves biotransformation in two phases: functionalization, which uses oxygen to form a reactive site (phase I detoxification), and conjugation, which results in addition of a water-soluble group to the reactive site (phase II biotransformation) resulting in formation of more water soluble compounds. The phase I detoxification is performed by monooxygenase enzymes including cytochrome P450s such as the ones differentially expressed in E+HS mice. In a study by Settivari et al. (2006), rats on an E+ diet for 5-d had higher expression of Cyp 450 genes involved in phase I detoxification while a down-regulation of genes involved in anti-oxidant pathways was observed. To date, there is little information about the hepatic metabolism and detoxification of ergot alkaloids in livestock. Cytochrome P450, CYP3A has been cited as the main P450 sub-family responsible for the metabolism of ergot alkaloids in other species, with N-dealkylation, mono- and dihydroxylation as the main oxidative processes carried out by these enzymes (Ball et al., 1992; Moubarak and Rosenkrans, 2000). Male mice on an E+ diet had 1.5 times more concentration of total microsomal Cyp 450 than female mice and this could be a source of potential variation in animal response to fescue toxins (Duringer et al., 2005). Up-regulation of Cyp 450 enzymes in E+HS mice (Table 10) would help in effective metabolism and detoxification of fescue toxins. It is interesting to note that mice fed an E+ diet under thermoneutral conditions (Bhusari et al., 2006) did not display changes in Cyp 450 gene expression when compared to mice fed E- under thermoneutral conditions.

Anti-oxidant gene expression changes

While enzymatic activity of cytochrome P450s are required for metabolism of xenobiotics, these reactions can lead to production of reactive oxygen species (ROS) and oxidative stress. During the course of the P450 catalytic cycle, P450s use H+ from NADPH to reduce O_2 leading to the production of H_2O_2 and superoxide anion radicals (Gonzalez, 2005). Expression of anti-oxidant gene metallothienin-1 (Mt-1; qPCR; Table 10) was increased by 160% in E+HS mice. This gene is involved in a wide range of activities from heavy metal detoxification to ROS scavenging (Sato and Kondoh, 2002). In many *in-vitro* experiments, Mt-1 has been shown to be a potent scavenger of hydroxyl radicals, protecting DNA from oxidative damage.

Expression of the carbonic anhydrase III gene (Car3; Table 10) was up-regulated in E+HS mice by 117%. The mammalian carbonic anhydrase reversibly hydrate carbon dioxide, thus generating both bicarbonate and hydrogen ions for maintenance of pH homeostasis. Two reactive sulfhydryl groups of Car3 can conjugate to glutathione through a disulfide link, a process termed S-glutathionylation. Car3 is rapidly glutathionylated *in-vivo* and *in-vitro* when cells are exposed to oxidative stresses, and it is one of the most carbonylated proteins in rodent liver (Kim et al., 2004). Overexpression of Car3 reduced steady-state levels of intracellular ROS, increased proliferation rate, and protected cells against H_2O_2-induced apoptosis (Raisanen et al., 1999).

Expression of copper chaperone for superoxide dismutase (Ccs) was up-regulated in E+HS mice by 51% (Table 10). The Ccs protein interacts with superoxide dismutase 1 (Sod1) and is responsible for copper incorporation into Sod1, which plays an important role in anti-oxidant metabolism (Wong et al., 2000). In mice with targeted disruption of

the Ccs gene, SOD1 enzyme activity is reduced and these mice had increased sensitivity to paraquat-induced oxidative stress (Wong et al., 2000).

Glutaredoxin 1 (Glrx1) was down-regulated in E+HS mice by 45%. Glutaredoxin proteins are low-molecular-weight proteins (9–12 kDa) with GSH-disulfide oxidoreductase activity. Glutaredoxins coupled to glutathione reductase are key players in anti-oxidant cellular processes based on the transfer of reducing equivalents from NADPH to disulfides via GSH (Porras et al., 2002). Cellular Glrx1 activity can be affected both directly (inactivation/inhibition) and indirectly by depletion of the second substrate, GSH, which occurs during oxidative stress and leads to accumulation of protein-SSG accumulation that could trigger apoptosis (Chrestensen et al., 2000).

The significant up-regulation of anti-oxidant gene expression (Table 10) could be aimed at counteracting the ROS and the resulting oxidative stress produced in the E+HS mice.

Conclusion

Genes coding for phase I detoxification enzymes are up-regulated in E+HS mouse liver. We observed an up-regulation of genes involved in the protection against reactive oxidant species, most probably to cope with the increase production of ROS from the phase I detoxification pathways. The most interesting result is that key genes involved in *de novo* lipogenesis and lipid transport are up-regulated. This is likely to have major consequence on hormone synthesis (steroids), circulating lipid concentrations and cellular lipid composition.

Table 10. List of genes differentially expressed in mice exposed exposed to E+HS vs E+TN

Gene [a] ID	Gene symbol	Gene Name	Gene ontology [b]	Ratio [c] (E+HS/E+TN)	*P*-value
Differentially expressed genes associated with lipid, cholesterol and carbohydrate metabolism					
11818	ApoH	Apolipoprotein H	Lipid transporter activity, phospholipids binding, defense response.	1.88, Up	0.005
21835	Thrsp	Thyroid hormone responsive SPOT14 homolog (Rattus)	Lipid metabolism, nucleus.	1.64, Up	0.009
104112	Acly	ATP citrate lyase	Transferase activity, lipid & acetyl-coA biosynthesis.	1.59, Up	0.02
14081	Acsl1	Acyl-CoA synthetase long-chain family member 1	Long-chain-fatty-acid-CoA ligase activity, lipid metabolism.	1.58, Up	0.01
18770	Pklr	Pyruvate kinase, liver and red blood cell	Transferase activity, glycolysis	1.51, Up	0.007
12759	Clu	Clusterin	Lipid metabolism, innate immune response, cell death.	1.50, Up	0.005
14104	Fasn	Fatty acid synthase	Transferase activity, lipid biosynthesis and metabolism.	1.48, Up	0.004
54135	Lsr	Lipolysis stimulated lipoprotein receptor	Transcription factor activity, lipid metabolism.	1.46, Up	0.006
68671	Pcyt2	Phosphate cytidylyltransferase 2, ethanolamine	Transferase activity, phospholipids metabolism.	1.37, Up	0.02
13167	Dbi	Diazepam binding inhibitor	Lipid binding, transport.	1.32, Up	0.01
238055	ApoB	Apolipoprotein B	Lipid, triacylglycerol mobilization.	0.61, Down	0.05
Differentially expressed genes associated with detoxification and anti-oxidant activity					
109754	Cyb5r3	Cytochrome b5 reductase 3	Oxidoreductase activity, xenobiotic metabolism.	2.93, Up	0.002
12350	Car3	Carbonic anhydrase 3	Metal ion binding, one-carbon metabolism.	2.17, Up	0.006
17748	Mt-1	Metallothionein 1	Metal ion binding, nitric oxide mediated signal transduction.	2.10, Up	0.002

12460	Ccs	Copper chaperone for superoxide dismutase	Metal ion, unfolded protein binding and superoxide metabolism.	1.51, Up	0.005
76279	Cyp2d26	Cytochrome P450, family 2, subfamily d, polypeptide 26	Monooxygenase activity, xenobiotic metabolism.	1.49, Up	0.001
13085	Cyp2a12	Cytochrome P450, family 2, subfamily a, polypeptide 12	Unspecific monooxygenase activity, drug metabolism.	1.42, Up	0.02
13850	Ephx2	Epoxide hydrolase 2, cytoplasmic	Metal ion binding, xenobiotic metabolism.	1.40, Up	0.01
18984	Por	P450 (cytochrome) oxidoreductase	Oxidoreductase activity, electron transport, xenobiotic metabolism.	0.70, Down	0.04
14263	Fmo5	Flavin containing monooxygenase 5	Monooxygenase activity, electron transport, xenobiotic metabolism.	0.60, Down	0.006
93692	Glrx	Glutaredoxin	Transferase activity, cellular redox activity.	0.55, Down	0.04
56388	Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	Monooxygenase activity, xenobiotic metabolism.	0.39, Down	0.002
Differentially expressed genes associated with stress, immune response and apoptosis					
625018	C4a	Complement component 4a	Inflammatory, innate-immune response.	1.91, Up	0.03
70356	St13	Suppression of tumorigenicity 13	Unfolded protein binding, protein folding.	1.38, Up	0.05
12630	Cfi	Complement factor I	Serine-type endopeptidase activity, innate immune response, proteolysis.	1.34, Up	0.02
18703	Pigr	Polymeric immunoglobulin receptor	Receptor activity.	1.30, Up	0.02
17195	Mbl2	Mannose binding lectin (C)	Sugar binding, phosphate transport, immune response.	0.65, Down	0.04
11796	Birc3	Baculoviral IAP repeat-containing 3	Suppressor of apoptosis.	0.44, Down	0.02
Differentially expressed genes associated with transport and protein biosynthesis					
27050	Rps3	Ribosomal protein S3	Structural constituent of ribosome, protein biosynthesis.	1.45, Up	0.04
15122	Hba-a1	Hemoglobin α, adult chain 1	Oxygen transporter activity.	1.43, Up	0.006
54192	Pbsn	Probasin	Transporter activity and transport.	1.42, Up	0.01

11938	Atp2a2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Nucleotide binding, transport.	1.37, Up	0.02
66211	Rpl3l	Ribosomal protein L3-like	Structural constituent of ribosome, protein biosynthesis.	1.35, Up	0.02
Differentially expressed genes associated with metabolism					
14317	Ftcd	Formiminotransferase cyclodeaminase	Lyase, transferase activity, folic acid metabolism.	1.77, Up	0.04
15445	Hpd	4-hydroxyphenylpyruvic acid dioxygenase	Oxidoreductase activity, tyrosine catabolism.	1.52, Up	0.008
20440	St6gal1	Beta galactoside alpha 2,6 sialyltransferase 1	Transferase activity, protein amino acid glycosylation.	1.49, Up	0.005
14085	Fah	Fumarylacetoacetate hydrolase	Tyrosine catabolism.	1.33, Up	0.01
67680	Sdhb	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	Succinate dehydrogenase (ubiquinone) activity, tricarboxylic acid cycle.	1.30, Up	0.03
11846	Arg1	Arginase 1, liver	Arginine metabolism.	0.65, Down	0.01
Differentially expressed genes associated with other cellular, biological processes					
13180	Pcbd1	Pterin 4 α carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 α (TCF1) 1	Transcription co-activator, protein homotetramerization.	1.89, Up	0.02
22644	Rnf103	Ring finger protein 103	Zinc ion binding, integral to membrane.	1.55, Up	0.0007

[a] Available: http://www.ncbi.nlm.nih.gov/entrez.
[b] Ontology classification of genes according to biological process, molecular function and cell location obtained using DAVID (http://david.niaid.nih.gov/david/).
[c] Ratio (E+HS/E+TN) is computed from the expression of gene in mice fed endophyte-infected (E+) diet at heat stress (E+HS) over its expression in mice fed E+ at thermoneutrality (TN). Up indicates up-regulated gene, while Down indicates down-regulated gene in E+HS mice.

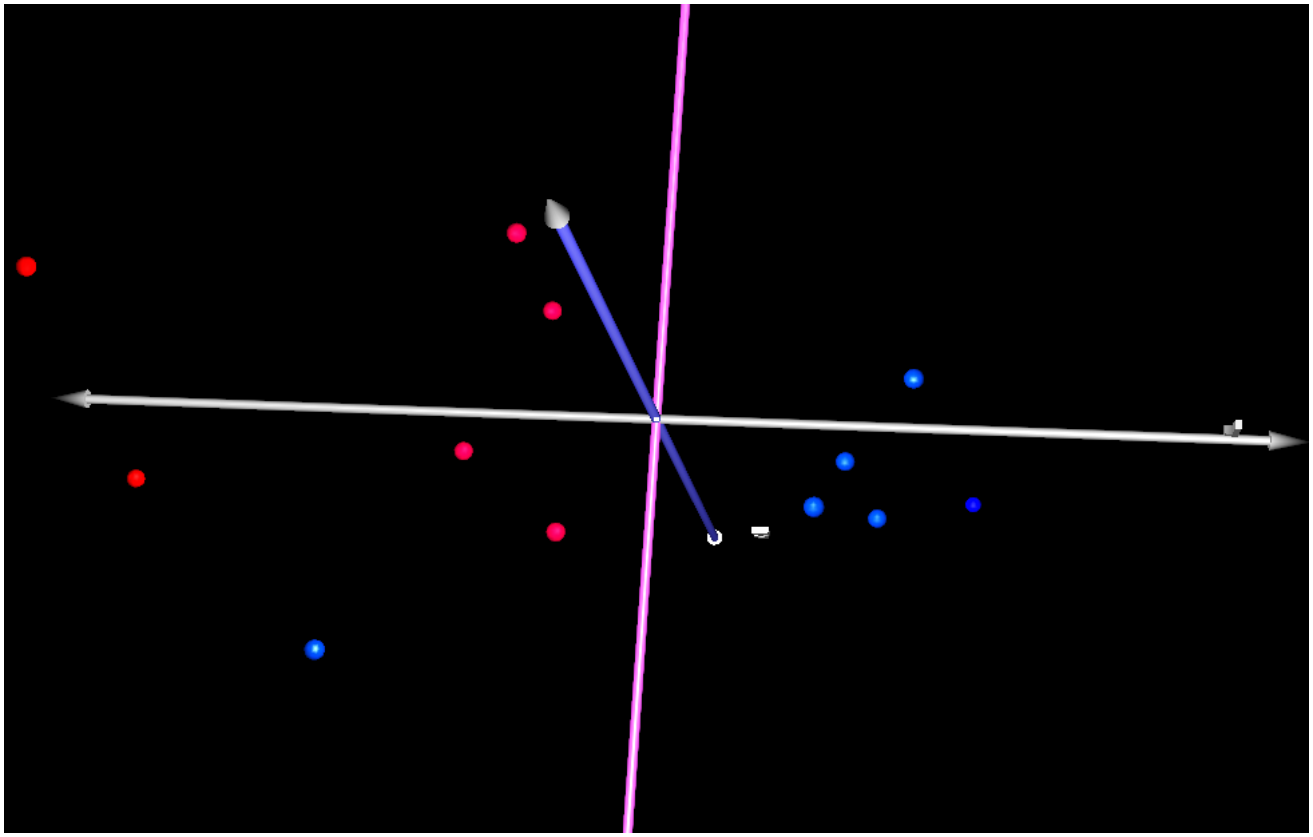

Figure 9: Principal component analysis (PCA) of E+HS and E+TN mice. Done to compare grouping of mice fed endophyte-infected (E+) diet under heat stress (HS; E+HS) against mice fed E+ under thermoneutral (TN; E+TN) condition. The red spheres in the figure indicate E+HS mice while E+TN mice are shown by blue spheres.

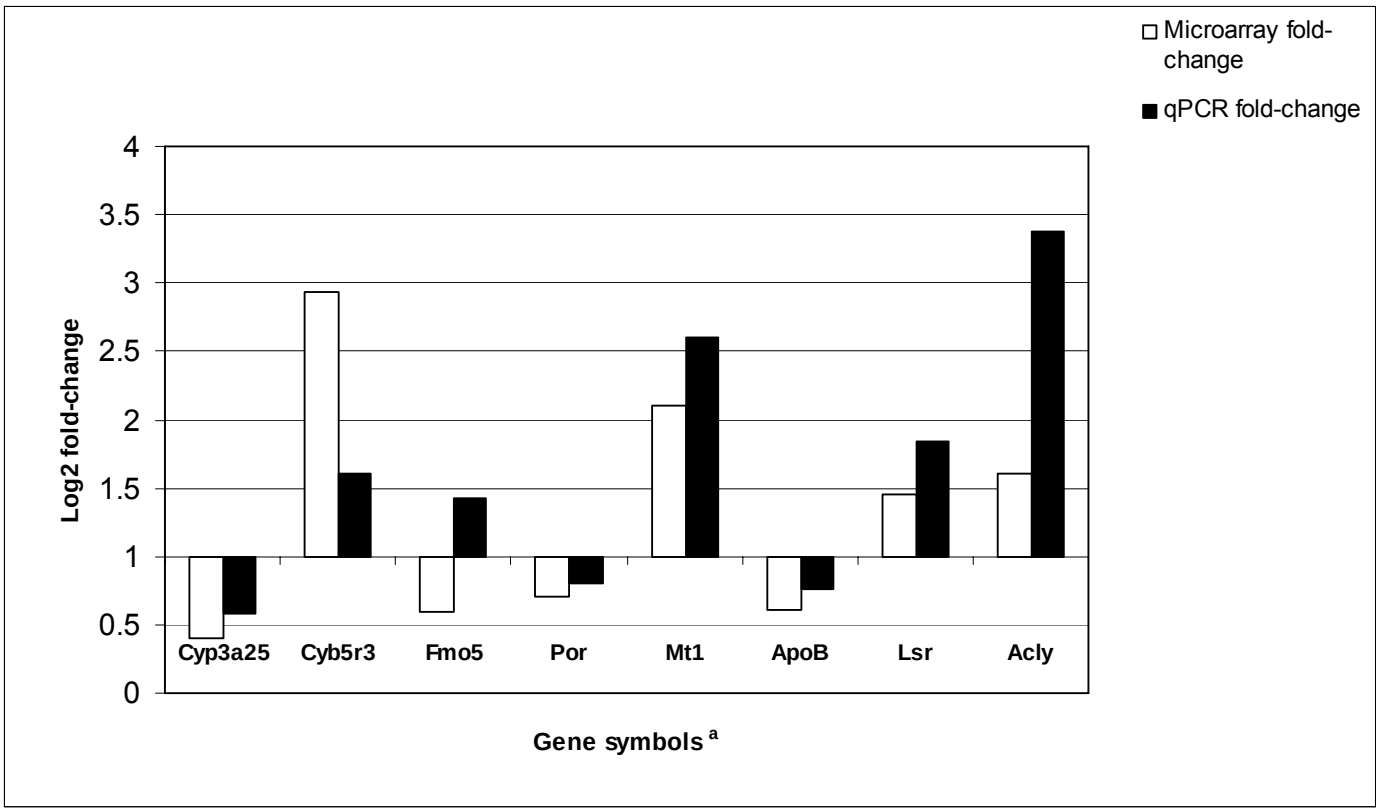

Figure 10: Expression analyses of eight genes showing log 2 of the ratio computed from the expression of gene in mice fed endophyte-infected (E+) diet at heat stress (HS) over its expression in mice fed endophyte-infected diet at thermoneutrality (TN). Solid and open bars represent qPCR or microarray data, respectively. [a] Additional information about gene symbols can be obtained from www.ncbi.nlm.nih.gov/entrez.

CHAPTER VI

SUMMARY

Comparison of gene expression changes due to E+, HS and E+HS

Mice fed an E+ diet under TN conditions resulted in changes in the expression of genes involved in the sex-steroid pathway while this pathway was not perturbed in mice exposed to HS (or) to E+HS treatments (Bhusari et al., 2006). Changes in expression of genes involved in the lipid and cholesterol metabolism pathways occurred in mice exposed to E+ and to E+HS treatment but not in mice exposed only to HS. Anti-oxidant gene expression changes occurred in mice exposed to HS and to E+HS, but not in E+ treated mice. Interestingly, gene expression changes involved in the detoxification pathway were seen only in mice exposed to combination of E+ and HS and these changes were not seen in mice exposed to only E+ or to HS. Thus, the combination of E+ and HS elicits a severe physiological response via increased detoxification of fescue toxins to effectively cope with increased stress. Pathways affected by various treatments are shown in Table 11.

Table 11. Genes involved in pathways changed in liver of mice treated with endophyte-infected (E+) diet, heat stress (HS) and the combination of E+ and HS

Pathways affected in mouse liver	**E+**	**HS**	**E+HS**
Sex-steroid metabolism	+	-	-
Lipid and cholesterol metabolism	+	-	+
De novo lipogenesis	-	-	+
Anti-oxidant	-	+	+
Detoxification	-	-	+

LITERATURE CITED

Abbaszade, I., T. Clarke, C. Park, and A. Payne. 1995. The mouse 3 beta-hydroxysteroid dehydrogenase multigene family includes two functionally distinct groups of proteins. Mol. Endocrinol. 9: 1214-1222.

Ahmad, S. 1995. Oxidative stress and antioxidant defenses in biology. Chapman & Hall, ©1995. New York.

Akhtar, M. K., S. L. Kelly, and M. A. Kaderbhai. 2005. Cytochrome b5 modulation of 17α-hydroxylase and 17-20 lyase (cyp17) activities in steroidogenesis. J. Endocrinol. 187: 267-274.

Alamer, M. A., and B. H. Erickson. 1990. Effect of fungus-infected fescue on testicular development and hormonal secretions in the beef bull. J. Anim. Sci. 68 (Suppl. 1): 402 (Abstr.).

Aldrich, C. G., J. A. Paterson, J. L. Tate, and M. Kerley. 1993. The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. J. Anim. Sci. 71: 164-170.

Al-Haidary, A., D. E. Spiers, G. E. Rottinghaus, G. B. Garner, and M. R. Ellersieck. 2001. Thermoregulatory ability of beef heifers following intake of endophyte-infected tall fescue during controlled heat challenge. J. Anim. Sci. 79: 1780-1788.

Arthur, J. R. 2000. The glutathione peroxidases. Cellular and Molecular Life Sciences (CMLS) 57: 1825-1835.

Bacon, C. W. 1995. Toxic endophyte-infected tall fescue and range grasses: Historic perspectives. J. Anim. Sci. 73: 861-870.

Bacon, C. W., J. K. Porter, J. D. Robbins, and E. S. Luttrell. 1977. *Epichloë typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34: 576-581.

Ball, S., G. Maurer, M. Zollinger, M. Ladona, and A. Vickers. 1992. Characterization of the cytochrome p-450 gene family responsible for the N-dealkylation of the ergot alkaloid CQA 206-291 in humans. Drug Metab. Dispos. 20: 56-63.

Barenton, B., and J. Pelletier. 1980. Prolactin, testicular growth and LH receptors in the ram following light and 2-br-agr-ergocryptine (cb-154) treatments. Biol. Reprod. 22: 781-790.

Beigneux, A. P., C. Kosinski, B. Gavino, J. D. Horton, W. C. Skarnes, and S. G. Young. 2004. ATP-citrate lyase deficiency in the mouse. J. Biol. Chem. 279: 9557-9564.

Bhusari, S., L. B. Hearne, D. A. Spiers, W. R. Lamberson, and E. Antoniou. 2006. Effect of fescue toxicosis on hepatic gene expression in mice. J. Anim. Sci. 84: 1600-1612.

Boling, J. A., L. D. Bunting, G. M. Davenport, J. L. Van der Veen, K. M. Meekins, N. W. Bradley, and R. E. Kohls. 1989. Physiological responses of cattle consuming tall fescue to environmental temperature and supplemental phenothiazine. J. Anim. Sci. 67: 2377-2385.

Bond, J., G. P. Lynch, D. J. Bolt, H. W. Hawk, G. Jackson, and R. J. Wall. 1988. Reproductive performance and lamb weight gains for ewes grazing fungus-infected tall fescue. Nutr. Rep. Int. 37: 1099-1115.

Bond, J., J. Powell, and B. Weinland. 1984a. Behavior of steers grazing several varieties of tall fescue during summer conditions. J. Agron. 76: 707–709.

Bond, J., J. B. Powell, D. J. Undersander, P. W. Moe, H. F. Tyrrell, and R. R. Oltjen. 1984b. Forage composition and growth and physiological characteristics of cattle grazing several varieties of tall fescue during summer conditions. J. Anim. Sci. 59: 584-593.

Brown, M. A., A. H. Brown, Jr, W. G. Jackson, and J. R. Miesner. 1993. Genotype x environment interactions in postweaning performance to yearling in Angus, Brahman, and reciprocal-cross calves. J. Anim. Sci. 71: 3273-3279.

Brown, N. M., A. S. Torres, P. E. Doan, and T. V. O'Halloran. 2004. Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu, Zn superoxide dismutase. Proc. Natl. Acad. Sci. USA 101: 5518-5523.

Browning, R., Jr, F. N. Schrick, F. N. Thompson, and T. Wakefield, Jr. 1998. Reproductive hormonal responses to ergotamine and ergonovine in cows during the luteal phase of the estrous cycle. J. Anim. Sci. 76: 1448-1454.

Buckner, R. C., J. B. Powell, and R. V. Frakes. 1979. Historical development. Fescue (ed.). Asa. p 1, Madison, WI.

Bulun, S. E., K. Takayama, T. Suzuki, H. Sasano, B. Yilmaz, and S. Sebastian. 2004. Organization of the human aromatase p450 (cyp19) gene. Semin. Reprod. Med. 22: 5-9.

Burke, J. M., D. E. Spiers, F. N. Kojima, G. A. Perry, B. E. Salfen, S. L. Wood, D. J. Patterson, M. F. Smith, M. C. Lucy, W. G. Jackson, and E. L. Piper. 2001. Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. Biol. Reprod. 65: 260-268.

Campbell, A. G., P. H. Mortimer, B. L. Smith, J. Clarke, and J. W. Ronaldson. 1975. Breeding for facial eczema resistance? In: Proceedings of the Ruakura Farmer's Conference. 62-64.

Caro, A. A., and A. I. Cederbaum. 2004. Oxidative stress, toxicology, and pharmacology of Cyp2e1. Ann. Rev. Pharmacol. Toxicol. 44: 27-42.

Cheeke, P. R. 1995. Endogenous toxins and mycotoxins in forage grasses and their effects on livestock. J. Anim. Sci. 73: 909-918.

Chirala, S. S., H. Chang, M. Matzuk, L. Abu-Elheiga, J. Mao, K. Mahon, M. Finegold, and S. J. Wakil. 2003. Fatty acid synthesis is essential in embryonic development: Fatty acid synthase null mutants and most of the heterozygotes die in utero. Proc. Natl. Acad. Sci. USA 100: 6358-6363.

Choudhary, D., I. Jansson, J. B. Schenkman, M. Sarfarazi, and I. Stoilov. 2003. Comparative expression profiling of 40 mouse cytochrome p450 genes in embryonic and adult tissues. Arch. Biochem. Biophys. 414: 91-100.

Chrestensen, C. A., D. W. Starke, and J. J. Mieyal. 2000. Acute cadmium exposure inactivates thioltransferase (glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J. Biol. Chem. 275: 26556-26565.

Churchill, G. A. 2002. Fundamentals of experimental design for cDNA microarrays. Nature Genetics 32: 490 - 495.

Cui, X., J. T. G. Hwang, J. Qiu, N. J. Blades, and G. A. Churchill. 2005. Improved statistical tests for differential gene expression by shrinking variance components estimates. Biostat. 6: 59-75.

Dalla Valle, L., S. Vianello, P. Belvedere, and L. Colombo. 2002. Rat cytochrome p450c17 gene transcription is initiated at different start sites in extraglandular and glandular tissues. J. Steroid Biochem. Mol. Biol. 82: 377-384.

Dennis, G., B. Sherman, D. Hosack, J. Yang, W. Gao, H. C. Lane, and R. Lempicki. 2003. David: Database for annotation, visualization, and integrated discovery. Genome Biology 4: P3.

Duringer, J. M., R. Lewis, L. Kuehn, T. Fleischmann, and A. M. Craig. 2005. Growth and hepatic in vitro metabolism of ergotamine in mice divergently selected for response to endophyte toxicity. Xenobiotica 35: 531 - 548.

DuTeaux, S. B., J. W. Newman, C. Morisseau, E. A. Fairbairn, K. Jelks, B. D. Hammock, and M. G. Miller. 2004. Epoxide hydrolases in the rat epididymis: Possible roles in xenobiotic and endogenous fatty acid metabolism. Toxicol. Sci. 78: 187-195.

Elsasser, T. H., and D. J. Bolt. 1987. Dopaminergic-like activity in toxic fescue alters prolactin but not growth hormone or thyroid stimulating hormone in ewes. Domest. Anim. Endocrinol. 4: 259-269.

Estienne, M. J., K. K. Schillo, B. P. Fitzgerald, S. M. Hileman, N. W. Bradely, and J. A. Bowling. 1990. Effects of endophyte-infected fescue on puberty onset and luteal activity in beef heifers. J. Anim. Sci. 68 (Suppl. 1): 567 (Abstr.).

Evans, R. R., R. L. Ivy, A. W. Groce, and W. Cox. 1989. Selenium supplementation for steers on fungus free and fungus infected fescue pastures (preliminary report). In: Proc. Tall Fescue Toxicosis Workshop, Atlanta, GA

Flanagan, S. W., A. J. Ryan, C. V. Gisolfi, and G. Kroemer. 1995. Tissue-specific hsp70 response in animals undergoing heat stress. Am. J. Physiol. Regul. Integ. Comp. Physiol. 268: R28-R32.

Forgione, M. A., N. Weiss, S. Heydrick, A. Cap, E. S. Klings, C. Bierl, R. T. Eberhardt, H. W. Farber, and J. Loscalzo. 2002. Cellular glutathione peroxidase deficiency and endothelial dysfunction. Am. J. Physiol. Heart Circ. Physiol. 282: H1255-1261.

Gaigg, B., T. B. F. Neergaard, R. Schneiter, J. K. Hansen, N. J. Fargeman, N. A. Jensen, J. R. Andersen, J. Friis, R. Sandhoff, H. D. Schroder, and J. Knudsen. 2001. Depletion of acyl-coenzyme a-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in Saccharomyces Cerevisiae. Mol. Biol. Cell 12: 1147-1160.

Garner, G. B., and C. N. Cornell. 1987. University of Missouri report on fescue research activities. (Preliminary report). In: Proc. Tall. Fescue Toxicosis Workshop, Memphis, TN

Godfrey, V. B., S. P. Washburn, E. J. Eisen, and B. H. Johnson. 1994. Effects of consuming endophyte-infected tall fescue on growth, reproduction and lactation in mice selected for high fecundity. Theriogenology 41: 1393-1409.

Goldstein, M., J. Y. Lew, A. Sauter, and A. Lieberman. 1980. The affinities of ergot compounds for dopamine agonist and dopamine antagonist reception sites. In: Advances in biochemical psychopharmacology No. 23. p 75. Raven Press, New York.

Gonzalez, F. J. 2005. Role of cytochromes p450 in chemical toxicity and oxidative stress: Studies with cyp2e1. Mutation Research/Fundamental and Molecular. Mechanisms of Mutagenesis Stress Responses 569: 101-110.

Gordon, C. J. 1993. Temperature regulation in laboratory rodents. Cambridge University Press, New York.

Gould, L. S., and W. D. Hohenboken. 1993. Differences between progeny of beef sires in susceptibility to fescue toxicosis. J. Anim. Sci. 71: 3025-3032.

Hemken, R., L. Bull, J. Boling, E. Kane, L. Bush, and R. Buckner. 1979. Summer fescue toxicosis in lactating dairy cows and sheep fed experimental strains of ryegrass-tall fescue hybrids. J. Anim. Sci. 49: 641-646.

Hemken, R. W., J. A. Boling, L. S. Bull, R. H. Hatton, R. C. Buckner, and L. P. Bush. 1981. Interaction of environmental temperature and anti-quality factors on the severity of summer fescue toxicosis. J. Anim. Sci. 52: 710-714.

Hildebrandt, A., and R. W. Estabrook. 1971. Evidence for the participation of cytochrome B5 in hepatic microsomal mixed-function oxidation reactions. Arch. Biochem. Biophys. 143: 66-79.

Hill, N. S., F. N. Thompson, J. A. Stuedemann, G. W. Rottinghaus, H. J. Ju, D. L. Dawe, and E. E. Hiatt. 2001. Ergot alkaloid transport across ruminant gastric tissues. J. Anim. Sci. 79:542–549.

Hohenboken, W. D., P. L. Berggren-Thomas, W. E. Beal, and W. H. McClure. 1991. Variation among Angus cows in response to endophyte-infected fescue seed in the diet, as related to their past calf production. J. Anim. Sci. 69: 85-90.

Hohenboken, W. D., and D. J. Blodgett. 1997. Growth and physiological responses to toxicosis in lines of mice selected for resistance or susceptibility to endophyte-infected tall fescue in the diet. J. Anim. Sci. 75: 2165-2173.

Horowitz, M. 2002. From molecular and cellular to integrative heat defense during exposure to chronic heat. Comp. Biochem. Physiol. Mol. Integr. Physiol. 131: 475-483.

Horowitz, M., L. Eli-Berchoer, I. Wapinski, N. Friedman, and E. Kodesh. 2004. Stress-related genomic responses during the course of heat acclimation and its association with ischemic-reperfusion cross-tolerance. J. Appl. Physiol. 97: 1496-1507.

Hoveland, C. S. 1993. Importance and economic significance of the acremonium endophytes to performance of animals and grass plant. Agric. Ecosys. Environ. 44: 3-12.

Hoveland, C. S., S. P. Schmidt, C. C. King, and E. M. Clark. 1984. Association of fungal endophyte with seasonal gains of beef steers grazing tall fescue pasture. In: H. Riley and a. D. Skelvag (ed.) proc. Eur. Grassl. Fed. p 382.

Janssen, G. B., R. B. Beems, L. H. Elvers, and G. J. A. Speijers. 2000. Subacute toxicity of α-ergocryptine in sprague-dawley rats. 2: Metabolic and hormonal changes. Food Chem. Toxicol. 38: 689-695.

Jones, J. M., J. C. Morrell, and S. J. Gould. 2000. Identification and characterization of haox1, haox2, and haox3, three human peroxisomal 2-hydroxy acid oxidases. J. Biol. Chem. 275: 12590-12597.

Jordan, E. R. 2003. Effects of heat stress on reproduction. J. Diary Sci. 86: (E. Suppl.):E104-E114.

Kang, Y. J. 1999. The antioxidant function of metallothionein in the heart. Proc. Soc. Exp. Biol. Med. 222: 263-273.

Kerr, M. K., and G. A. Churchill. 2001. Statistical design and the analysis of gene expression microarray data. Genet. Res. 77: 123-128.

Kim, G., T.-H. Lee, P. Wetzel, C. Geers, M. A. Robinson, T. G. Myers, J. W. Owens, N. B. Wehr, M. W. Eckhaus, G. Gros, A. Wynshaw-Boris, and R. L. Levine. 2004. Carbonic anhydrase III is not required in the mouse for normal growth, development, and life span. Mol. Cell. Biol. 24: 9942-9947.

Kinlaw, W. B., J. L. Church, J. Harmon, and C. N. Mariash. 1995. Direct evidence for a role of the ``spot 14'' protein in the regulation of lipid synthesis. J. Biol. Chem. 270: 16615-16618.

Kregel, K. 2002. Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolarance. J. App. Physiol. 92: 2177-2186.

Kurian, J. R., S. U. Bajad, J. L. Miller, N. A. Chin, and L. A. Trepanier. 2004. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. J. Pharmacol. Exp. Ther. 311: 1171-1178.

Kwak, M.-K., N. Wakabayashi, J. L. Greenlaw, M. Yamamoto, and T. W. Kensler. 2003. Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. Mol. Cell. Biol. 23: 8786-8794.

Laws, G. M., T. R. Skopek, M. V. Reddy, R. D. Storer, and W. E. Glaab. 2001. Detection of DNA adducts using a quantitative long pcr technique and the fluorogenic 5' nuclease assay (Taqman(r)). Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 484: 3-18.

Lee, S.-H., A. Dobrzyn, P. Dobrzyn, S. M. Rahman, M. Miyazaki, and J. M. Ntambi. 2004. Lack of stearoyl-coA desaturase 1 upregulates basal thermogenesis but causes hypothermia in a cold environment. J. Lipid Res. 45: 1674-1682.

Levadoux-Martin, M., J. E. Hesketh, J. H. Beattie, and H. M. Wallace. 2001. Influence of metallothionein-1 localization on its function. Biochem. J. 355: 473-479.

Lipham, L. B., F. N. Thompson, J. A. Stuedemann, and J. L. Sartin. 1989. Effects of metoclopramide on steers grazing endophyte-infected fescue. J. Anim. Sci. 67: 1090-1097.

Liu, J., H. C. Yeo, E. Overvik-Douki, T. Hagen, S. J. Doniger, D. W. Chu, G. A. Brooks, and B. N. Ames. 2000. Chronically and acutely exercised rats: Biomarkers of oxidative stress and endogenous antioxidants. J. Appl. Physiol. 89: 21-28.

Loizou, J. I., S. F. El-Khamisy, A. Zlatanou, D. J. Moore, D. W. Chan, J. Qin, S. Sarno, F. Meggio, L. A. Pinna, and K. W. Caldecott. 2004. The protein kinase ck2 facilitates repair of chromosomal DNA single-strand breaks. Cell 117: 17-28.

Martinez, L. O., S. Jacquet, J.-P. Esteve, C. Rolland, E. Cabezon, E. Champagne, T. Pineau, V. Georgeaud, J. E. Walker, F. Terce, X. Collet, B. Perret, and R. Barbaras. 2003. Ectopic β-chain of ATP synthase is an apolipoprotein a-i receptor in hepatic HDL endocytosis. Nature 421: 75-79.

McMurphy, W. E., K. S. Lusby, S. C. Smith, S. H. Muntz, and C. A. Strasia. 1990. Steer performance on tall fescue pasture. J. Prod. Agric 3: 100.

Mesli, S., S. Javorschi, A. M. Berard, M. Landry, H. Priddle, D. Kivlichan, A. J. H. Smith, F. T. Yen, B. E. Bihain, and M. Darmon. 2004. Distribution of the lipolysis stimulated receptor in adult and embryonic murine tissues and lethality of Lsr-/- embryos at 12.5 to 14.5 days of gestation. Eur. J. Biochem. 271: 3103-3114.

Miles, C. O., A. L. Wilkins, R. T. Gallagher, A. D. Hawkes, S. C. Munday, and N. R. Towers. 1992. Synthesis and tremorgenicity of paxitriols and lolitriol: Possible biosynthetic precursors of lolitrem. B. J. Agric. Food Chem. 40: 234-236.

Miller, B. F., K. L. Armstrong, L. A. Wilson, W. D. Hohenboken, and R. G. Saacke. 1994. Variation among inbred and linecross mice in response to fescue toxicosis. J. Anim. Sci. 72: 2896-2904.

Monroe, J. L., D. L. Cross, L. W. Hudson, D. M. Henricks, S. W. Kennedy, and W. C. Bridges. 1988. Effect of selenium and endophyte-contaminated fescue on performance and reproduction in mares. J. Equine Vet. Sci. 8: 148.

Morris, C. A., N. R. Towers, A. G. Campbell, J. J. Meyer, C. Wesslink, and M. Wheeler. 1989. Response achieved in Romney flocks selected for or against susceptibility to facial eczema. N. Z. J. Agric. Res 32: 379.

Moubarak, A. S., and J. Rosenkrans, C. F. 2000. Hepatic metabolism of ergot alkaloids in beef cattle by cytochrome p450. Biochem. Biophys. Res. Commun. 274: 746-749.

Murray, J. I., M. L. Whitfield, N. D. Trinklein, R. M. Myers, P. O. Brown, and D. Botstein. 2004. Diverse and specific gene expression responses to stresses in cultured human cells. Mol. Biol. Cell 15: 2361-2374.

Mustonen, M., M. Poutanen, S. Kellokumpu, Y. de Launoit, V. Isomaa, R. Vihko, and P. Vihko. 1998. Mouse 17 beta-hydroxysteroid dehydrogenase type 2 mRNA is predominantly expressed in hepatocytes and in surface epithelial cells of the gastrointestinal and urinary tracts. J. Mol. Endocrinol. 20: 67-74.

Nihsen, M. E., E. L. Piper, C. P. West, R. J. Crawford, Jr., T. M. Denard, Z. B. Johnson, C. A. Roberts, D. A. Spiers, and C. F. Rosenkrans, Jr. 2004. Growth rate and

physiology of steers grazing tall fescue inoculated with novel endophytes. J. Anim. Sci. 82: 878-883.

Ntambi, J. M. 1999. Regulation of stearoyl-coa desaturase by polyunsaturated fatty acids and cholesterol. J. Lipid Res. 40: 1549-1558.

Ntambi, J. M., and M. Miyazaki. 2004. Regulation of stearoyl-coA desaturases and role in metabolism. Prog. Lipid Res. 43: 91-104.

Nutting, D. F., E. A. Tolley, L. A. Toth, S. D. Ballard, and M. A. Brown. 1992. Serum amylase activity and calcium and magnesium concentrations in young cattle grazing fescue and bermuda grass pastures. Am. J. Vet. Res. 53: 834-839.

Oda, M. N., J. K. Bielicki, T. T. Ho, T. Berger, E. M. Rubin, and T. M. Forte. 2002. Paraoxonase 1 overexpression in mice and its effect on high-density lipoproteins. Biochem. Biophys. Res. Commun. 290: 921-927.

Ohsawa, I., K. Nishimaki, C. Yasuda, K. Kamino, and S. Ohta. 2003. Deficiency in a mitochondrial aldehyde dehydrogenase increases vulnerability to oxidative stress in pc12 cells. J. Neurochem. 84: 1110-1117.

Ohta, S., I. Ohsawa, K. Kamino, F. Ando, and H. Shimokata. 2004. Mitochondrial aldh2 deficiency as an oxidative stress. Ann. NY. Acad. Sci. 1011: 36-44.

Osborn, T. G., S. P. Schmidt, D. N. Marple, C. H. Rahe, and J. R. Steenstra. 1992. Effect of consuming fungus-infected and fungus-free tall fescue and ergotamine tartrate on selected physiological variables of cattle in environmentally controlled conditions. J. Anim. Sci. 70: 2501-2509.

Oufara, S., H. Barre, J. L. Rouanet, and J. Chatonnet. 1987. Adaptation to extreme ambient temperatures in cold-acclimated gerbils and mice. Am. J. Physiol. 253: R39-45.

Pan, M., A. I. Cederbaum, Y.-L. Zhang, H. N. Ginsberg, K. J. Williams, and E. A. Fisher. 2004. Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein b degradation and VLDL production. J. Clin. Invest. 113: 1277-1287.

Parkes, H. A., E. Preston, D. Wilks, M. Ballesteros, L. Carpenter, L. Wood, E. W. Kraegen, S. Furler, and G. J. Cooney. 2006. Over-expression of acyl-coa synthetase-1 increases lipid deposition in hepatic (Hepg2) cells and rodent liver in vivo. Am. J. Physiol. Endocrinol. Metab. 112.

Paterson, J., C. Forcherio, B. Larson, M. Samford, and M. Kerley. 1995. The effects of fescue toxicosis on beef cattle productivity. J. Anim. Sci. 73: 889-898.

Peters, C. W., K. N. Grigsby, C. G. Aldrich, J. A. Paterson, R. J. Lipsey, M. S. Kerley, and G. B. Garner. 1992. Performance, forage utilization, and ergovaline consumption by beef cows grazing endophyte fungus-infected tall fescue,

endophyte fungus-free tall fescue, or orchardgrass pastures. J. Anim. Sci. 70: 1550-1561.

Peters-Volleberg, G. W. M., R. B. Beems, and G. J. A. Speijers. 1996. Subacute toxicity of ergometrine maleate in rats. Food Chem. Toxicol. 34: 951-958.

Piper, E. L. 1989. Liver function and ryegrass staggers. N. Z. Vet. J 37: 173.

Polz, E., and G. M. Kostner. 1979a. The binding of β2-glycoprotein-i to human serum lipoproteins : Distribution among density fractions. FEBS Letters 102: 183-186.

Polz, E., and G. M. Kostner. 1979b. Binding of β2-glycoprotein-i to intralipid: Determination of the dissociation constant. Biochem. Biophys. Res. Commun. 90: 1305-1312.

Porras, P., J. R. Pedrajas, E. Martinez-Galisteo, C. Alicia Padilla, C. Johansson, A. Holmgren, and J. Antonio Barcena. 2002. Glutaredoxins catalyze the reduction of glutathione by dihydrolipoamide with high efficiency. Biochem. Biophys. Res. Commun. 295: 1046-1051.

Porter, J. K. 1995. Analysis of endophyte toxins: Fescue and other grasses toxic to livestock. J. Anim. Sci. 73: 871-880.

Porter, J. K., and F. N. Thompson, Jr. 1992. Effects of fescue toxicosis on reproduction in livestock. J. Anim. Sci. 70: 1594-1603.

Quackenbush, J. 2002. Microarray data normalization and transformation. Nature Genetics 32: 496-501.

Raisanen, S., P. Lehenkari, M. Tasanen, P. Rahikila, P. L. Harkonen, and H. K. Vaananen. 1999. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. FASEB J. 13: 513-522.

Rennie, P. S., N. Bruchovsky, K. J. Leco, P. C. Sheppard, S. A. McQueen, H. Cheng, R. Snoek, A. Hamel, M. E. Bock, and B. S. MacDonald. 1993. Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. Mol. Endocrinol. 7: 23-36.

Rhodes, M. T., J. A. Paterson, M. S. Kerley, H. E. Garner, and M. H. Laughlin. 1991. Reduced blood flow to peripheral and core body tissues in sheep and cattle induced by endophyte-infected tall fescue. J. Anim. Sci. 69: 2033.

Rottinghaus, G. E., L. M. Schultz, P. F. Ross, and N. S. Hill. 1993. An HPLC method for the detection of ergot in ground and pelleted feeds. J. Vet. Diagn. Invest. 5: 242-247.

Saal, L., C. Troein, J. Vallon-Christersson, S. Gruvberger, A. Borg, and C. Peterson. 2002. Bioarray software environment (base): A platform for comprehensive

management and analysis of microarray data. Genome Biology 3: software0003.0001 - software0003.0006.

Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush. 2003. Tm4: A free, open-source system for microarray data management and analysis. Biotechniques 34: 374-378.

Sandberg, M. B., M. Bloksgaard, D. Duran-Sandoval, C. Duval, B. Staels, and S. Mandrup. 2005. The gene encoding Acyl-coA-binding protein is subject to metabolic regulation by both sterol regulatory element-binding protein and peroxisome proliferator-activated receptor α in hepatocytes. J. Biol. Chem. 280: 5258-5266.

Santos, J. H., B. S. Mandavilli, and B. Van Houten. 2002. Measuring oxidative mtDNA damage and repair using quantitative PCR. In: W. C. Copeland (ed.) Mitochondrial DNA, methods and protocols. Methods in molecular biology No. 197. p 159-176. Humana Press.

Sato, M., and M. Kondoh. 2002. Recent studies on metallothionein: Protection against toxicity of heavy metals and oxygen free radicals. Tohoku J. Exp. Med. 196: 9-22.

Schmidt, S. P., D. A. Danilson, J. A. Holliman, H. W. Grimes, and W. B. Webster. 1986. Fescue fungus suppresses growth and reproduction in replacement beef heifers. Highlights Agric. Res. Ala. Agric. Exp. Stn. Auburn, Ala. 33: 15.

Schmidt, S. P., and T. G. Osborn. 1993. Effects of endophyte-infected tall fescue on animal performance. Agri. Ecosys. Environ. 44: 233-262.

Schriner, S. E., N. J. Linford, G. M. Martin, P. Treuting, C. E. Ogburn, M. Emond, P. E. Coskun, W. Ladiges, N. Wolf, H. Van Remmen, D. C. Wallace, and P. S. Rabinovitch. 2005. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 308: 1909-1911.

Schwimmer, H., L. Eli-Berchoer, and M. Horowitz. 2006. Acclimatory-phase specificity of gene expression during the course of heat acclimation and superimposed hypohydration in the rat hypthalamus. J. Appl. Physiol. 100: 1992-2003.

Seath, D. M., C. A. Lassiter, J. W. Rust, M. Cole, and G. M. Bastin. 1956. Comparative value of kentucky blue grass, kentucky 31 fescue, orchard grass, and bromegrass as pastures for milk cows. 1. How kinds of grasses affected persistence of milk production, tdn yield, and body weight. J. Dairy Sci. 39: 574-580.

Settivari, R. S., S. Bhusari, T. Evans, P. A. Eichen, L. B. Hearne, E. Antoniou, and D. E. Spiers. 2006. Genomic analysis of the impact of fescue toxicosis on hepatic function. J. Anim. Sci. 84.

Sharma, T. P., D. Blache, C. E. Roselli, and G. B. Martin. 2004. Distribution of aromatase activity in brain and peripheral tissues of male sheep: Effect of nutrition. Reprod. Fertil. Dev. 16: 709-715.

Shelby, R., and L. Dalrymple. 1987. Incidence and distribution of the tall fescue endophyte in the United States. Plant Disease 71: 783-785.

Smith, C., J. Carney, P. Starke-Reed, C. Oliver, E. Stadtman, R. Floyd, and W. Markesbery. 1991. Excess brain protein oxidation and enzyme dysfunction in normal aging and in alzheimer disease. Proc. Natl. Acad. Sci. USA 88: 10540-10543.

Smith, M. S. 1980. Role of prolactin in regulating gonadotropin secretion and gonad function in female rats. Fed Proc. 39: 2571-2576.

Smith, V. G., T. W. Beck, E. M. Convey, and H. A. Tucker. 1974. Bovine serum prolactin, growth hormone, cortisol and milk yield after ergotamine. Neuroendocrinology 15: 172-181.

Soncin, F., X. Zhang, B. Chu, X. Wang, A. Asea, M. Ann Stevenson, D. B. Sacks, and S. K. Calderwood. 2003. Transcriptional activity and DNA binding of heat shock factor-1 involve phosphorylation on threonine 142 by ck2. Biochem. Biophys. Res. Commun. 303: 700-706.

Sonna, L. A., J. Fujita, S. L. Gaffin, and C. M. Lilly. 2002. Effects of heat and cold stress on mammalian gene expression. J. Appl. Physiol. 92.

Sonna, L. A., B. C. Wenger, S. Flinn, H. K. Sheldon, M. N. Sawka, and C. M. Lilly. 2004. Exertional heat injury and gene expression changes: A DNA microarray analysis study. J. Appl. Physiol. 96: 1943-1953.

Spiers, D. E., Q. Zhang, P. A. Eichen, G. E. Rottinghaus, G. B. Garner, and M. R. Ellersieck. 1995. Temperature-dependent responses of rats to ergovaline derived from endophyte-infected tall fescue. J. Anim. Sci. 73: 1954-1961.

Spiers, D. E., P. A. Eichen, and G. E. Rottinghaus. 2005. A model of fescue toxicosis: Responses of rats to intake of endophyte-infected tall fescue. J. Anim. Sci. 83: 1423-1434.

St-Pierre, N. R., B. Cobanov, and G. Schnitkey. 2003. Economic losses from heat stress by us livestock industries. J. Dairy Sci. 86: E52-77.

Strahan, S. R., R. W. Hemken, J. A. Jackson, R. C. Buckner, L. P. Bush, and M. R. Siegel. 1987. Performance of lactating dairy cows fed tall fescue forage. J. Dairy Sci. 70: 1228-1234.

Stuedemann, J. A., and C. S. Hoveland. 1988. Fescue endophyte: History and impact on animal agriculture. J. Prod. Agricul. 1: 39-44.

Stuedemann, J. A., T. S. Rumsey, J. Bond, S. R. Wilkinson, L. P. Bush, D. J. Williams, and A. B. Caudle. 1985a. Association of blood cholesterol with occurrence of fat necrosis in cows and tall fescue summer toxicosis in steers. Am. J. Vet. Res. 46: 1990-1995.

Stuedemann, J. A., T. S. Rumsey, J. Bond, S. R. Wilkinson, L. P. Bush, D. J. Williams, and A. B. Caudle. 1985b. Association of blood cholesterol with occurrence of fat necrosis in cows and tall fescue summer toxicosis in steers. Am. J. Vet. Res. 46: 1990-1995.

Stuedemann, J. A., N. S. Hill, F. N. Thompson, R. A. Fayrer-Hoskin, W. P. Hay, D. L. Dawe, D. H. Seman, and S. A. Martin. 1998. Urinary and biliary excretion of ergot alkaloids from steers that grazed endophyhte-infected tall fescue. J. Anim. Sci.76:2146–2154.

Swinnen, J. V., P. Alen, W. Heyns, and G. Verhoeven. 1998. Identification of diazepam-binding inhibitor/acyl-coa-binding protein as a sterol regulatory element-binding protein-responsive gene. J. Biol. Chem. 273: 19938-19944.

Thompson, F. N., and J. A. Stuedemann. 1993. Pathophysiology of fescue toxicosis. Agri. Ecosys. Environ. 44: 263-281.

Thompson, F. N., J. A. Stuedemann, J. L. Sartin, D. P. Belesky, and O. J. Devine. 1987. Selected hormonal changes with summer fescue toxicosis. J. Anim. Sci. 65: 727-733.

Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA 98: 5116-5121.

Varney, D. R., C. J. Kappes, S. L. Jones, R. Newsome, M. R. Siegel, and P. M. Zavos. 1988. The effect of feeding tall fescue seed infected by acremonium coenophialum on pregnancy and parturition in female rats. Comp. Biochem. Physiol. 89C: 315-320.

Vianello, S., M. R. Waterman, L. D. Valle, and L. Colombo. 1997. Developmentally regulated expression and activity of 17α-hydroxylase/c-17, 20-lyase cytochrome p450 in rat liver. Endocrinology 138: 3166-3174.

Wagner, C. R., T. M. Howell, W. D. Hohenboken, and D. J. Blodgett. 2000. Impacts of an endophyte-infected fescue seed diet on traits of mouse lines divergently selected for response to toxins in endophyte-infected fescue. J. Anim. Sci. 78: 1191.

Walls, J. R., and D. R. Jacobson. 1970. Skin temperature and blood flow in the tail of dairy heifers administered extracts of toxic tall fescue. J. Anim. Sci. 30: 420.

Wolfinger, R. D., G. Gibson, E. D. Wolfinger, L. Bennett, H. Hamadeh, P. Bushel, C. Afshari, and R. S. Paules. 2001. Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. 8: 625-637.

Wong, J. S., and S. G. Sarjeet. 2002. Gene expression changes induced in mouse liver by di(2-ethylhexyl) phthalate. Toxicol. Appl. Pharmacol. 185: 180-196.

Wong, P. C., D. Waggoner, J. R. Subramaniam, L. Tessarollo, T. B. Bartnikas, V. C. Culotta, D. L. Price, J. Rothstein, and J. D. Gitlin. 2000. Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc. Natl. Acad. Sci. USA 97: 2886-2891.

Wu, H., K. Kerr, and G. Churchill. 2003. Maanova: A software package for the analysis of spotted cDNA microarray experiments. In the analysis of gene expression data: An overview of methods and software. p 313–431. Springer, New York.

Yen, F. T., M. Masson, N. Clossais-Besnard, P. Andre, J.-M. Grosset, L. Bougueleret, J.-B. Dumas, O. Guerassimenko, and B. E. Bihain. 1999. Molecular cloning of a lipolysis-stimulated remnant receptor expressed in the liver. J. Biol. Chem. 274: 13390-13398.

Zavos, P. M., T. M. McShane, K. L. Evans, N. W. Bradley, R. W. Hemken, and M. R. Siegel. 1988a. Effect of feeding tall fescue seed infected with endophytic fungus (*acremonium coenophialium*) on the estrous cycle in CD-1 mice. Drug Chem. Toxicol. 11: 443-450.

Zavos, P. M., B. Salim, J. Jackson, J. A., D. R. Varney, M. R. Siegel, and R. W. Hemken. 1986. Effect of feeding tall fescue seed infected by endophytic fungus on reproductive performance in male rats. Theriogenology 25: 281-290.

Zavos, P. M., D. R. Varney, R. M. Hemken, M. R. Siegel, and L. P. Bush. 1988b. Fertilization rates and embryonic development in CD-1 mice fed fungal endophyte-infected tall fescue seed. Theriogenology 30: 461-468.

Zavos, P. M., D. R. Varney, J. A. Jackson, M. R. Siegel, L. P. Bush, and R. W. Hemken. 1987. Effect of feeding fungal endophyte (*acremonium coenophialum*) - infected tall fescue seed on reproductive performance in cd-1 mice through continuous breeding. Theriogenology 27: 549-559.

Zhang, H. J., V. J. Drake, J. P. Morrison, L. W. Oberley, and K. C. Kregel. 2002. Molecular biology of thermoregulation: Selected contribution: Differential expression of stress-related genes with aging and hyperthermia. J. Appl. Physiol. 92: 1762-1769.

Zhang, H. J., L. Xu, V. J. Drake, L. Xie, L. W. Oberley, and K. C. Kregel. 2003. Heat-induced liver injury in old rats is associated with exaggerated oxidative stress and altered transcription factor activation. FASEB J. 17: 2293-2295.

Zhu, Q., G. W. Anderson, G. T. Mucha, E. J. Parks, J. K. Metkowski, and C. N. Mariash. 2005. The spot 14 protein is required for de novo lipid synthesis in the lactating mammary gland. Endocrinology 146: 3343-3350.

Ziegler, D. M. 2002. An overview of the mechanism, substrate specificities, and structure of FMOs. Drug Metabolism Reviews 34: 503-511.

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