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Research article

Bovine hepatic differential gene expression in response to perennial ryegrass staggers

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Abstract

"Perennial ryegrass staggers" is a neurological condition characterized by muscular tremors and ataxia in livestock that ingest the indole diterpene lolitrem B from endophyte-infected perennial ryegrass. While the neurotoxic mechanism of action of ryegrass staggers has been defined, little is known about metabolic and other molecular processes that lolitrem B may affect in livestock. The objective of this study was to characterize differential gene expression in the liver of animals fed lolitrem B-containing feed over an extended period of time. Eighteen steers were fed one of three rations (n =6/group) containing lolitrem B (247, 1,554 or 2,256 parts per billion (ppb, DM basis)) over 64 days in a double-blind study. Microarray and confirmatory qPCR were performed to evaluate the hepatic gene expression profile from biopsies taken on days 0 and 64. Widespread perturbation of gene expression was observed in all groups receiving lolitrem B, with a total of 152 differential genes identified (FDR $\leq$ 0.05). This suggests that chronic exposure to lolitrem B, even at levels below the current threshold of toxicity (2,000 ppb LB), can perturb many genes, biological processes and pathways. Gene ontology (GO) and Kyoto Encyclopedia of Genes and genomes (KEGG) pathway analyses indicated that many of these genes were categorized under lipid/steroid biosynthesis/metabolism and oxidation-reduction. Specifically, genes involved in the biosynthesis pathway of ceramide, a sphingolipid molecule (*ACSS2*, *LASS6* and *SCD*) and changes in neurosignaling through alteration of nitric oxide synthase activity (*ARG1* and *GPX4*) were up-regulated. Future work should focus on the overall balance between ceramide and its metabolites and antioxidants/oxidants in a variety of body matrices in animals with perennial ryegrass staggers, to determine how these compounds contribute to the overall etiology of this disease.

Keywords

ceramide, microarray, lolitrem B, cattle

Introduction

The symbiosis between perennial ryegrass (*Loliium perenne*) and the endophytic fungus *Neotyphodium lolii* results in increased grass fitness through enhancement of insect resistance, drought tolerance and seed production. Unfortunately, this endophyte produces the mycotoxin lolitrem B, which is an indole-diterpenoid neurotoxin responsible for the syndrome known as 'perennial ryegrass staggers' (Gallagher and Hawkes, 1986; Gallagher *et al.*, 1984). Perennial ryegrass staggers has been reported most frequently in New Zealand and the Pacific Northwest region of the United States, and has been observed in sheep, cattle, horses, deer and alpacas (Cheeke, 1995; diMenna *et al.*, 2012). The main clinical sign of ryegrass staggers is muscular tremoring, which is exaggerated by external stimuli (Fisher *et al.*, 2004; Gallagher and Hawkes, 1986). As the toxicosis worsens, animals lose coordination and develop a stiff gait. They may collapse and have convulsions, leaving them susceptible to dehydration, starvation and attack by predators. A dietary threshold of toxicity level of 1,800-2,000 ppb lolitrem B in both cattle and sheep has been established for this disease syndrome which appears over 1-3 weeks (Blythe *et al.*, 2007; Tor-Agbidye *et al.*, 2001). At present, there is no effective treatment for perennial ryegrass staggers, except removing animals from exposure to lolitrem B-containing feed (Gilruth, 1906). When contaminated feed is removed, animals typically regain normal neuromuscular function within 4-7 days.

The mechanism of action for inducing perennial ryegrass staggers involves inhibition of large conductance calcium-activated potassium (BK) channels by lolitrem B, as observed in human cells (Dalziel *et al.*, 2005; Imlach *et al.*, 2011) and in rodent models (Imlach *et al.*, 2008, 2009). Blood chemistry and necropsy findings in camels fed perennial ryegrass straw over 56 days (Alabdouli *et al.*, 2014) and in horses fed perennial ryegrass seed and hay for 10-14 days

(Johnstone and Mayhew, 2013) show alterations in kidney function may occur as well. To our knowledge, changes in gene expression in response to lolitrem B-containing feed in livestock, specifically those targeting neurological and metabolic/detoxification pathways, has not been studied. Thus, our objective was to use microarray and qPCR in a pilot study to identify differentially expressed genes in the liver of 18 steers fed perennial ryegrass straw containing varying concentrations of lolitrem B over 64 days.

Materials and methods

Animal work and lolitrem B feeding

All procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee, Oregon State University (OSU). Steers (*Bos taurus*, breed: Angus cross, 7-8 months old, average body weight = 295 kg) were purchased from a feedlot in Oregon, castrated and treated with an anthelminthic prior to shipping. Upon arrival, animals were initially placed on pasture with grain supplementation for 10 days with free access to the barn for shelter. Following this initial acclimation period, animals were randomly separated into pens of six steers each. Steers were allowed two weeks to adjust to chopped perennial ryegrass straw (0 ppb lolitrem B) prior to day 0 of the study. Rations were formulated with 247 (low), 1,554 (mid) or 2,256 (high) ppb lolitrem B (all values were based on dry matter and were tested at the Endophyte Service Laboratory, OSU (Hovermale and Craig, 2001)); each pen of steers was fed one ration via a double-blind format twice daily *ad libitum* for 64 days. Total perennial ryegrass straw and orts from each pen were collected and weights recorded daily in order to adjust the next day's feeding for each pen. Orts were pooled each week by pen and analyzed for lolitrem B concentration. During the study, an error was made in the feed for the high group which was unintentionally given a lower ration averaging 302 ppb for days 19-26. Once the error was discovered, animals were placed back on high feed for the remainder of the trial. In addition to perennial ryegrass straw, steers were also provided with a concentrate, CHS Beef Grower 20, at 0.9 kg/steer/day. Salt blocks were available *ad libitum*, in addition to water. Body weight and the ratio of straw intake versus body weight of each feeding group were monitored weekly throughout the experimental period. Animals were clinically evaluated for ryegrass staggers using a scoring system modified from previous work (Fisher *et al.*, 2004; Galey *et al.*, 1991). A score of 0 = no clinical signs; a score = 1 was resting tremors of the head and/or neck; and a score of 2 = resting tremors of the head, neck and/or body, incoordination with handling, and marked stiffness of gait.

Liver tissue sampling

Liver biopsies were performed on days 0 and 64 on each steer at the 11th intercostal space, where a line from the tuber coxa to the olecraneon intersects. Steers were prepared by clipping the hair from a six cm square around the specified location, followed by a three step surgical prep. A number 15 scalpel blade was used to make the initial incision through the skin. A 14-gauge Biopty needle in a Biopty gun (Bard Urological, Covington, GA) was inserted into the incision aiming in a cranioventral direction. The needle was sharply thrust into the liver parenchyma to a depth of approximately eight cm before the gun was triggered. After biopsy, samples were immediately placed into vials containing RNAlater solution (Ambion Inc., Austin, TX) on ice and transferred within 6 h to the lab and stored at -20ºC.

RNA extraction

The RNAlater-stored samples were homogenized with a Precellys 24 Bead Mill Homogenizer (Omni International, Kennesaw, GA). An RNeasy mini kit (Qiagen, Valencia, CA) was used to extract total RNA. RNA quantification and purification were analyzed by a

ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The RNA sample aliquots were kept at −80°C until use. RNA was checked for quality and integrity using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

Microarray study design and protocol

A total of 36 bovine microarray chips (containing 26,303 genes and 194,712 probe sets, Bovine Gene v1 Array (BovGene-1_0-v1), Affymetrix, Santa Clara, CA) were used, with six biological replicates per group for day 0 and the same for day 64. Each RNA sample was hybridized on one chip. Microarray assays were performed at the Center for Genome Research and Biocomputing, OSU. All RNA samples were randomized to make labeling and hybridization reactions across chips as uniform as possible. Labeled target cDNA was prepared from 125 ng of bovine liver total RNA using the NuGen Applause WT-Amp Plus ST RNA amplification system kit protocol and encore biotin module V2 (NuGEN Technologies, Inc., San Carlos, CA). Fragmented cDNA (4.45 µg) from each sample was hybridized for 18 hours to an array using the NuGEN hybridization protocol as follows: pre-hybridize chip for 10-15 min, heat cocktail at 99ºC for 2 min, 45ºC for 5 min, spin 5 min, remove pre-hybridization buffer from array, then fill array with 200 µL hybridization cocktail; hybridize for 18 h at 45ºC, rotating at 60 rpm. NuGen Applause WT-Amp Plus ST labeling was done without stop to the end of the SPIA amplification protocol in addition to purification and quantification. The samples were frozen at -80ºC overnight and then thawed, completing the fragmentation, labeling and QC. QC showed cDNA traces similar to previously run samples. Washing, staining and scanning followed the GeneChip Expression Wash, Stain and Scan Manual for Cartridge Arrays protocol (Affymetrix), using AGCC software (version 3.0, Affymetrix) with a Fluidics Station 450, and a GeneChip Scanner 3000 with autoloader (Affymetrix). Image processing and data extraction were performed using AGCC software. The chip definition file (CDF) was obtained from Affymetrix. The file included information on probeset ID, gene ID, gene symbol and gene annotation description.

Statistical analyses of microarray data

Microarray raw data were transferred to log (base 2) intensities and normalized using the Affymetrix Expression Console software. The Robust Multichip Average (RMA) normalization process involved background adjustment and quantile normalization (Irizarry *et al.*, 2003). The normalized log intensities were approximately normally distributed (Supplemental Figure 1). Using the R/Bioconductor Limma package (Smyth, 2004), a linear model was fit to the normalized log intensities and a moderated paired *t*-test was used to compare the mean log intensities between day 0 (control) and day 64 (lolitrem B) in each of the three feeding groups (low, mid and high). The results were summarized using *P*-values and adjusted *P*-values. The adjusted *P*-values were computed using Benjamini and Hochberg's method (Benjamini and Hochberg, 1995), in which the false discovery rates (FDR) are evaluated corresponding to the *P*-values. Supplemental Figure S2 shows the distribution of *P*-values for the paired comparisons in each of the three groups (low, mid and high).

Hierarchical cluster analysis

Hierarchical cluster analysis was computed by R using the Complete Linkage Clustering algorithm (Perruchet, 1983).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses

Differential gene symbols from each group (low, mid and high) were imported into DAVID Bioinformatics Resource 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (Huang *et al.*, 2009a, 2009b). Using the functional annotation chart tool from the DAVID database, enriched GO terms and KEGG pathways were computed. The Elim method in topGo computes the statistical significance of a parent node dependent on the significance of its children by Fisher's Exact Test. Nodes are significant if the *P*-value is smaller than a previously defined threshold (Alexa *et al.*, 2006). The *P*-value was adjusted with the Benjamini-Hochberg procedure for controlling the FDR (Benjamini and Hochberg, 1995). KEGG biological pathways (http://www.genome.jp/kegg/pathway.html, (Kanehisa and Goto, 2000) were used to elucidate molecular interactions and networks involved in biological processes such as biosynthesis, metabolism and the cell cycle.

qPCR validation

The selection of five candidates for further confirmatory qPCR was based several factors: 1) differentially expressed genes with the lowest FDR (Supplementary Table 1); 2) GO and KEGG analyses; and 3) interest in xenobiotic metabolism. Available resources limited the number of genes we could follow up with qPCR. The extracted RNA sample aliquots were subjected to reverse transcription (RT) using a SuperScript III first-strand synthesis kit (Invitrogen, Carlsbad, CA). The reaction conditions were: 10 µL reaction mix, two µL SuperScript III reverse transcriptase and RNaseOUT mix, one µg template RNA from each sample and DEPC-treated water to bring the total reaction volume to 20 µl. The reaction set-up was: 25ºC x 10 min, 50ºC x 30 min, 85ºC x 5 min, then chilled on ice; finally, one µL of *E.coli* RNase H (two units) was added and incubated at 37ºC for 20 min. The cDNA products from RT were stored at -20ºC until qPCR was performed. Quantification of the selected genes (see Supplemental Table S2 for primer sequences, purchased from Invitrogen) was performed by amplification of the cDNA products, as described above, using an Applied Biosystems 7500 Fast real-time thermocycler (Applied Biosystems, Foster City, CA). A DyNAmo flash SYBR green qPCR kit was purchased from New England Biolabs (Rockford, IL). The cDNA products were diluted five times; two µL of the diluted cDNA was loaded into each well of a 96-well microplate. Total volume of each well was 20 µL. Samples were run in duplicate. The intra-assay variation for each sample duplicate was very small. Optimized amplification conditions in each well were: 10 µL reaction master mix, 4 x 10^{-7}mol/L primers, 2.5 x 10^{-3} mol/L $MgCl_2$, 2 x 10^{-4} mol/L dNTPs, and 0.3 x ROX passive reference dye. Reaction set-up included: 95°C x 7 min, 95°C x 15 s, 60°C x 30 s, 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (reference gene) and measured separately (non-multiplex). Cqs of the no-template controls (NTC) were 35.80-38.71 or non-detectable for all targeted and reference genes. Cqs of targets for all samples ranged from 21.54 to 27.49. Cqs of GADPH from all qPCR experiments ranged from 18.18-20.51. The amplicon specificity was performed via dissociation curve analysis. Tms for targets ranged from 79.1-84.9°C; Tms for GAPDH ranged from 83.7-84.0°C. All the amplicon lengths were 100-297 bp. 7500 Fast System SDS software (version 1.4, Applied Biosystems) and the relative quantitative mode were used for data collection. Due to the characteristics of relative quantitation PCR without standard curves, there were no PCR efficiencies displayed in the results. However, preliminary validation tests showed that the efficiencies of the target and endogenous control amplifications were approximately equal. The relative quantity of each gene expression was calculated as $2^{\wedge-\Delta\Delta Cq}$, ΔCq = Cq (target)-Cq (GAPDH), $\Delta\Delta Cq = \Delta Cq$ (day 60)-ΔCq (day 0). A paired *t*-test was utilized for qPCR statistics to determine the difference between day 64 and the corresponding control (day 0). One-way ANOVA with Dunn's post-test was used to determine the difference among the three groups (low, mid and high) with (day 64) or without (day 0) lolitrem B

intake. qPCR data was analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

Hepatic differential gene expression in steers fed lolitrem B-containing perennial ryegrass straw

There were no significant differences in gene expression among the three groups before lolitrem B intake (day 0) (all false discovery rate (FDR) values > 0.05). Analysis of the overall effect of lolitrem B across all three groups (low, mid and high) on day 64 compared to their corresponding control (day 0) revealed significantly altered expression (FDR ≤ 0.05) for 1,574 genes. The number of total differential, up- and down-regulated genes in the three groups are summarized in a Venn Diagram (Figure 1). 152 of the 1,574 differentially expressed genes were shared amongst all three groups (FDR ≤ 0.05). Among them, 122 genes were up-regulated and 30 genes were down-regulated. However, no significant difference in gene expression between the three groups on Day 64 was detected (all FDR were > 0.05).

Enriched gene ontology (GO) categories are shown in Table 1, including oxidation reduction and biosynthesis and metabolism of different fats (lipids, cholesterol, steroids and sterols). Consistently, enriched KEGG pathways in all three groups were also related to the biosynthesis of different lipids (fatty acids, steroid, hormones and terpenoids) (Table 2). Both microarray and qPCR data analyses had the same gene expression pattern for the five selected genes: they were up-regulated in all three groups (low, mid and high), as compared to their corresponding controls (day 0). qPCR found that two genes (*SCD* (delta-9 desaturase) and *ACSS2* (acetyl-coenzyme A synthetase)) were up-regulated by ≥ seven-fold and three genes (*LASS6* (longevity-assurance homolog 6), *ARG1* (arginase), *GPX4* (glutathione peroxidase 4)) were up-regulated by ≥ two-fold in all three groups as compared to the controls (Table 3). Figure 2 shows a ceramide synthesis pathway (a cause for neurodegeneration) in which three (*SCD*, *ACSS2* and *LASS6*) of the five differentially expressed genes are involved.

Dose-specific effects

Presentation of clinical signs in the animals was complicated by the unintentional “washout” in the high group from days 19-26. The veterinary neurologist evaluating the animals restarted the clock and counted from day 27 in the high group in defining the date of onset of clinical signs. After 22 days of being on consistent high feed, two out of six animals presented with a score of 1 on our clinical scale. At 31 days, four out of six animals had a score of 1; at 35 days, four out of six animals had a score of 1 and one animal had progressed to ataxia and a score of 2, for a total of five out of six animals affected. This continued until the end of the study (37 days on 2,256 ppb lolitrem B). The mid group, which received a continuous dose of 1,554 ppb lolitrem B, began showing a score of 1 in two out of six animals on day 52. On day 53, this progressed to three out of six animals with a score of 1; by day 61 four out of six animals had a score of 1 which was maintained until the end of the study (64 days). The low group (247 ppb lolitrem B) showed no clinical signs for the entire study.

The gene ontology analyses revealed dose-specific effects on biological processes (Table 1) and pathways (Table 2). For example, cholesterol and steroid biosynthesis pathways appeared to be deregulated only in mid and high groups, but terpenoid backbone biosynthesis processes were affected only in the mid group. However, tests for individual genes based on the

microarray or the qPCR data detected no significant differentially expressed genes among the three groups (low, mid and high) at day 64 (FDR > 0.05).

Discussion

It was hypothesized prior to this study that the clinical signs of perennial ryegrass staggers would be associated with corollary changes in genes involved in neurological and metabolic/detoxification pathways. Microarray analysis elucidated differentially expressed genes from specific physiological pathways and enriched GO categories including biosynthesis and metabolism of various lipids (unsaturated fatty acids, steroids and steroid hormones) and oxidation reduction (Tables 1 and 2). Altered gene expression occurred in the liver of all steers that consumed endophyte-infected perennial ryegrass straw for 64 days containing lolitrem B (ranging from 247-2,256 ppb), even though the clinical signs of perennial ryegrass staggers occurred only in mid (day 52 for 1,554 ppb lolitrem B) and high (day 22 for 2,256 ppb lolitrem B) groups. The threshold of toxicity of lolitrem B in cattle fed perennial ryegrass straw over a shorter period of time (28 days) was ~ 2,000 ppb (Blythe *et al.*, 2007), below which no clinical evidence of ryegrass staggers disease was observed. Thus, this study indicated that the threshold of toxicity for lolitrem B-induced ryegrass staggers is lower in animals exposed to lolitrem B for longer time periods, which is corroborated by the observation of differential gene expression in the liver of cattle consuming all given doses of lolitrem B.

The lack of a dose-dependent effect on the specific genes measured via qPCR may be due to several factors. First, the "washout" period from days 19-26 in the high group may have softened the effects of lolitrem B on differential gene expression regarding metabolic functions in liver cells, making the mid and high groups less distinguished from each other. Liver biopsies were taken on day 64, so the high group was exposed for 38 days to 2,256 ppb lolitrem B but the difference in long-term accumulation of the toxin could play a role in the lack of a dose response. Secondly, statistical power (only six biological replicates were used per group) may not have been able to account for the individual variability seen in this disease. A need for a larger sample size was noted in a feeding trial with horses (n = 7/group) fed lolitrem B-containing perennial ryegrass which observed: 1) substantial individual animal variability in severity of clinical signs within a group of animals fed the same amount of lolitrem B; and 2) lack of correlation of serum lolitrem B with clinical signs (Johnstone *et al.*, 2012). Thus, it appears that lolitrem B may elicit a range of both clinical and physiological responses which should be taken into account when designing future studies by including larger sample sizes. Additional time points or dosages may help refine these effects as well. Lastly, comparison to the control group would have been another way to analyze for differential gene expression, but, in that case, one would be sacrificing controlling for individual animal differences which appears to be significant for ryegrass staggers.

Importantly, both microarray and qPCR results demonstrated that a two month exposure to lolitrem B-containing perennial ryegrass straw up-regulated expression of genes associated with the ceramide biosynthesis pathway in liver tissue (Figure 2). This outcome provides biological plausibility for an additional mechanism of action for the clinical signs observed in cases of perennial ryegrass staggers, and mirrors findings of alteration in sphingolipid metabolism for other mycotoxins including fumonisins and ochratoxins (Doi and Uetsuka, 2011; Stockmann-Juvala and Savolainen, 2008), albeit through varying mechanisms, including some with nephro- and hepatotoxicities (Bucci *et al.*, 1998; Mathur *et al.*, 2001). Ceramide is a sphingolipid molecule composed of sphingosine and a saturated or unsaturated long-chain fatty acyl group; ceramides with phosphorylcholine esterified at the 1-OH position

make up the sphingomyelins, the most abundant sphingolipid group in mammalian tissues, particularly in cell membranes (Devlin, 2010). In addition, ceramide acts as a bioactive lipid, playing a role in signaling and regulation of cell-mediated stress responses including apoptosis and cell senescence, differentiation and cell-cycle arrest (Hannun and Obeid, 2008).

Amongst the five ~~potential~~ most differentially expressed genes identified in liver tissue from our study(Table 3), three (*ACSS2, LASS6* and *SCD*) are involved in the pathway of ceramide biosynthesis. The gene *ACSS2* encodes a catalytic enzyme for the synthesis of fatty acyl-CoA (EC 6.2.1.1), which is a precursor to ceramide (Luong *et al.*, 2000), while *LASS6* (EC 2.3.1.24) is a key ceramide synthase in the ceramide biosynthesis salvage pathway (Kitatani *et al.*, 2008). The third, *SCD* (EC 1.14.19.1), utilizes O_2 and electrons from reduced cytochrome b_5 to catalyze the insertion of a double bond into fatty acyl-CoA substrates, such as palmitoyl-CoA and stearoyl-CoA, which are components of the ceramide synthesis pathway (Kitatani *et al.*, 2008; Oshino *et al.*, 1971). It was reported that in *SCD* gene-deficient mice, ceramide synthesis was reduced by decreasing palmitoyl-CoA synthesis (Dobrzyn *et al.*, 2005). Further, oxidation, as well as fatty acid desaturases such as *SCD*, plays a critical role in adjusting the biosynthesis and biophysical properties of ceramide and its downstream metabolites (Behrouzian and Buist, 2002). This may explain why oxidation-reduction was an enriched GO category (Table 1).

The liver isoform of arginase, over-expressed *ARG1* (EC 3.5.3.1) can affect nitric oxide (NO) synthase activity and NO-dependent smooth muscle relaxation by depleting the substrate pool of L-arginine that would otherwise be available to NO synthase as part of the urea cycle (Delker *et al.*, 2010). Once NO synthase is affected, its product NO is decreased which may affect vasodilation (Devlin, 2010) or neurotransmission (Prast and Philippu, 2001). Alternatively, increased *ARG1* could indicate a drive to eliminate the increased nitrogenous waste lolitrem B presents as urea in hepatic cells (Devlin, 2010).

Lastly, *GPX4* (EC 1.11.1.12), or glutathione peroxide 4, catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and lipid peroxides and is thus involved in lipid metabolism (Muller *et al.*, 2007). Depletion of GPX4 has been shown to cause neuronal degeneration *in vivo* and *ex vivo* through apoptosis triggered by increased oxidative stress (Seiler *et al.*, 2008). Arginase activation was found to have a negative relationship with GPX activity in mice (Malleske *et al.*, 2006). Other research using the rat kidney KNRK cell line found that inactivation of GPX activity triggered induction of *GPX* in order to restore intracellular levels of this enzyme (Dobashi *et al.*, 2001). The up-regulation of the *GPX4* gene might therefore be feedback of reduced NO due to overexpressed *ARG1*. Although the *GPX4*-coded enzyme is generally considered an antioxidant, ceramide accumulation is involved in oxidative stress in the nervous system (Cutler *et al.*, 2004). Upon examining the brain of sheep and cattle with staggers, it was concluded that the lesions in the brain described are not regarded as pathognomonic of ryegrass staggers but probably arise from a number of factors, which may include disturbed neuronal metabolism, neuronal exhaustion and repeated anoxic insults (Mason, 1968). The overall balance between antioxidants and oxidants in the neural system of animals with perennial ryegrass staggers would be of interest to investigate in future work.

It is not surprising that steroid and steroid hormone biosynthesis and retinol metabolism pathways were significant after KEGG analysis (Table 2), as steroids in animals are biologically produced from terpenoids and terpenoid precursors (Schroepfer, 1982); lolitrem

B is also a diterpenoid molecule. Deregulation of steroid pathways and androgen/estrogen metabolism is likely the cause of hormone homeostasis disruption associated with reproduction and lactation abnormalities, which are other clinical signs associated with perennial ryegrass staggers (Prestidge, 1993).

Conclusions

Five differentially expressed genes in hepatic tissue whose main roles fall under ceramide metabolism and oxidation-reduction provided additional ~~a~~ plausible mechanisms of action for the etiology of perennial ryegrass staggers which should be followed-up and confirmed in future biomonitoring studies of protein surrogates. Overexpression of these genes would cause an abundance of ceramide, neural apoptosis and neurodegeneration, and deregulated neurotransmission and signaling, which could contribute to tremor and ataxia, the clinical signs of ryegrass toxicosis. The genes identified that are involved in the ceramide synthesis pathway were consistent with the enriched GO terms and KEGG pathways, in which lipid biosynthesis and metabolism were prominent. Since ceramide is a bioactive lipid, how it interacts with other sphingolipids or other lipids such as steroids in the neural system remains to be seen. Additional studies will focus on the quantitative analysis of ceramide and its metabolites, including the possibility of lolitrem B-ceramide analogues (Bartók *et al.*, 2013), in different tissues/matrices (e.g. blood, liver, brain and fat) of animals presenting with ryegrass staggers to further define the mechanism(s) of toxicity of lolitrem B. In addition, using lolitrem B as an inhibitor of the specific gene-encoded enzymes involved in the biosynthesis and metabolism of ceramide delineated in this study will help us understand the larger picture of how lolitrem B interacts with physiological processes including cell growth, differentiation and death, and altered neurotransmission/neurodegeneration throughout the entire animal.

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Table 1 Enriched Gene Ontology (GO) categories in cattle fed lolitrem B-containing perennial ryegrass straw for 64 days.

Enriched GO terms	Low*	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
	No. of genes			**% Total**			***P*-value**			**FDR**		
oxidation reduction	14	16	21	9.9	17.6	10.4	$3.1\ e^{-4}$	$6.3\ e^{-8}$	$1.9\ e^{-6}$	$1.2\ e^{-1}$	$2.8\ e^{-6}$	$1.1\ e^{-3}$
lipid biosynthetic process	5	13	11	3.5	14.3	12.2	$5.4\ e^{-2}$	$8.6\ e^{-11}$	$2.9\ e^{-5}$	1.0	$6.2\ e^{-9}$	$8.8\ e^{-3}$
cholesterol biosynthetic process	0	8	5	0	8.8	2.5	~	$3.3\ e^{-12}$	$5.0\ e^{-5}$	~	$1.2\ e^{-9}$	$1.0\ e^{-2}$
sterol biosynthetic process	0	8	5	0	8.8	2.5	~	$7.9\ e^{-12}$	$7.8\ e^{-5}$	~	$1.4\ e^{-9}$	$1.0\ e^{-2}$
steroid biosynthetic process	0	9	6	0	9.9	3.0	~	$3.5\ e^{-11}$	$1.0\ e^{-4}$	~	$4.1\ e^{-9}$	$1.2\ e^{-2}$
cholesterol metabolic process	0	9	6	0	9.9	3.0	~	$6.4\ e^{-11}$	$1.4\ e^{-4}$	~	$5.8\ e^{-9}$	$1.4\ e^{-2}$
sterol metabolic process	0	9	7	0	9.9	3.5	~	$1.4\ e^{-10}$	$2.2\ e^{-4}$	~	$8.2\ e^{-9}$	$1.9\ e^{-2}$
steroid metabolic process	0	9	7	0	9.9	3.5	~	$1.6\ e^{-8}$	$4.6\ e^{-4}$	~	$8.4\ e^{-7}$	$3.4\ e^{-2}$

*Low (247 ppb), Mid (1,554 ppb) or High (2,256 ppb) indicates concentration of lolitrem B in the perennial ryegrass straw fed over 64 days to steers, n=6/group

No. of genes = number of genes from Supplementary Table 1 with FDR ≤ 0.01

% total = ratio of genes involved in a gene ontology category relative to the total number of differentially expressed genes for each group

P-value was computed using the functional annotation chart tool from the DAVID database (http://david.abcc.ncifcrf.gov/home.jsp)

FDR is an adjusted *P*-value (Benjamini *et al.*, 1995)

Table 2 Pathway deregulation identified from the Kyoto Encyclopedia for Genes and Genomes (KEGG) in cattle fed lolitrem B-containing perennial ryegrass straw for 64 days.

Pathway Name	Low*		Mid		High	
	***P*-value**	**FDR**	***P*-value**	**FDR**	***P*-value**	**FDR**
steroid biosynthesis	~	~	$9.0\ e^{-8}$	$3.9\ e^{-6}$	$6.4\ e^{-5}$	$2.6\ e^{-3}$
biosynthesis of unsaturated fatty acids	$1.5\ e^{-2}$	20	$4.3\ e^{-4}$	$9.5\ e^{-3}$	$3.2\ e^{-2}$	$2.6\ e^{-2}$
androgen and estrogen metabolism	$3.5\ e^{-6}$	$2.0\ e^{-4}$	$9.2\ e^{-4}$	$1.3\ e^{-3}$	$2.5\ e^{-5}$	$2.1\ e^{-3}$
steroid hormone biosynthesis	$1.8\ e^{-5}$	$5.0\ e^{-4}$	$2.3\ e^{-3}$	$2.5\ e^{-2}$	$1.2\ e^{-4}$	$3.4\ e^{-3}$
porphyrin and chlorophyll metabolism	$1.8\ e^{-3}$	$3.3\ e^{-2}$	$9.2\ e^{-4}$	$1.3\ e^{-2}$	$5.5\ e^{-3}$	11
retinol metabolism	$5.6\ e^{-2}$	$4.2\ e^{-1}$	$3.4\ e^{-3}$	$2.9\ e^{-2}$	$1.9\ e^{-2}$	23
terpenoid backbone biosynthesis	~	~	$4.4\ e^{-3}$	$3.2\ e^{-2}$	~	~

*Low (247 ppb), Mid (1,554 ppb) or High (2,256 ppb) indicates concentration of lolitrem B in the perennial ryegrass straw fed over 64 days to steers, n=6/group

Pathway name was created from KEGG pathways; details of all KEGG pathways are available at http://www.genome.jp/keg/pathways.html

P-value was computed using the functional annotation chart tool from the DAVID database (http://david.abcc.ncifcrf.gov/home.jsp)

FDR is an adjusted *P*-value (Benjamini *et al.*, 1995)

Table 3 Comparison of differentially expressed genes between microarray and qPCR analysis in the liver of cattle exposed to lolitrem B-containing perennial ryegrass straw for 64 days.

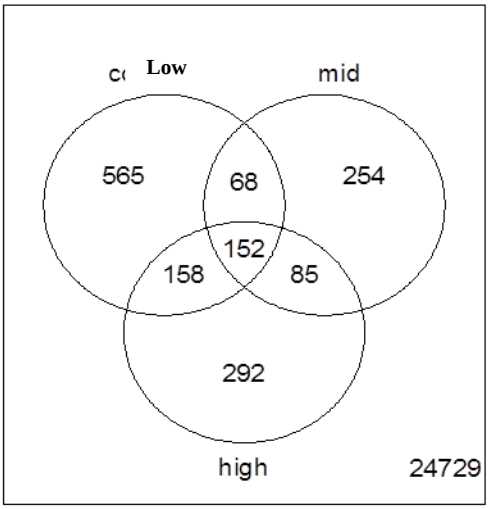
			Low[a]			*Mid*			*High*		
Gene ID[b]	**Symbol**	**Definition**	**Microarray**		**qPCR**[e]	**Microarray**		**qPCR**	**Microarray**		**qPCR**
			FC[c]	*FDR*[d]	*FC*	*FC*	*FDR*	*FC*	*FC*	*FDR*	*FC*
12821369	*SCD*	stearoyl-CoA desaturase	10.97	8.74 e^{-6}	21.94	11.09	1.17 e^{-5}	23.17	12.67	4.85 e^{-6}	30.95
12714225	*ACSS2*	acyl-CoA synthetase short-chain family member 2	4.23	5.09 e^{-5}	8.84	4.84	1.90 e^{-5}	8.77	5.79	6.74 e^{-6}	7.61
12877228	*GPX4*	glutathione peroxidase 4	2.12	2.37 e^{-4}	2.43	1.64	7.86 e^{-3}	2.04	1.91	8.76 e^{-4}	2.37
12897200	*ARG1*	arginase, liver	1.86	3.54 e^{-3}	3.21	1.73	8.97 e^{-3}	1.93	1.69	1.15 e^{-2}	2.25
12784416	*LASS6*	LAG1 homolog, ceramide synthase 6	1.71	9.54 e^{-3}	3.75	1.83	6.08 e^{-3}	2.01	1.84	1.44 e^{-2}	2.44

[a]Low, Mid and High correspond to liver samples taken from cattle fed perennial ryegrass containing 247, 1,554 and 2,256 ppb lolitrem B, respectively.
[b]Affymetrix Bovine Gene v1 Array probe ID
[c]FC = fold change of group on day 64 as compared to their corresponding control (day 0)
[d]FDR is an adjusted *P*-value (Benjamini *et al.*, 1995)
[e]All qPCR *P*-values < 0.05, n = 4 biological replicates in each group

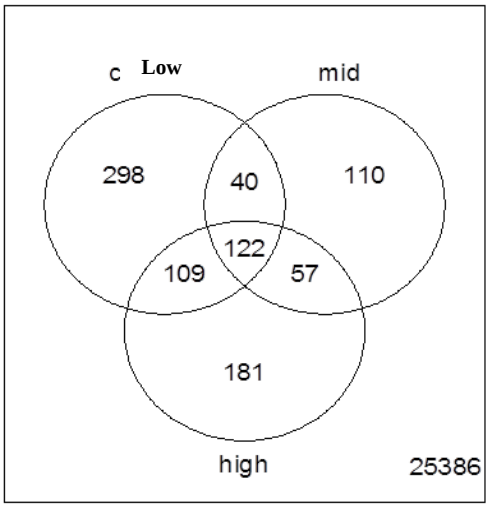
Annotations for figures

Figure 1 Venn diagrams showing total (a), up-regulated (b) and down-regulated (c) differentially expressed genes in low, mid and high groups on day 64 compared with their corresponding control (day 0) (FDR ≤ 0.05).

A: Total differentially expressed genes



B: Up-regulated genes



C: Down-regulated genes

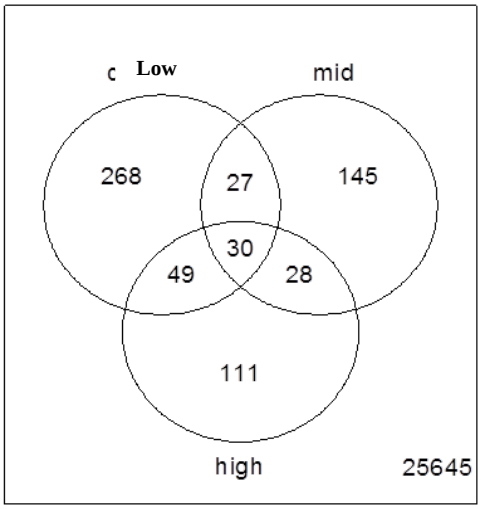


Figure 2 Up-regulated genes (bold) differentially expressed in the liver of cattle fed lolitrem B-containing ryegrass for 64 days involved in the ceramide biosynthesis pathway. The *ACSS2* gene encodes a catalytic enzyme that plays a role in the synthesis of fatty acyl-CoA; *LASS6* is a ceramide synthase in the ceramide biosynthesis salvage pathway; *SCD* belongs to the fatty acid desaturase family which is involved in ceramide synthesis, and also functions as terminal components of the liver microsomal stearyl-CoA desaturase system that utilizes O_2

and electrons from reduced cytochrome b5 to catalyze the insertion of a double bond into palmitoyl-CoA.