Ergovaline in tall fescue and its effect on health, milk quality, biochemical parameters, oxidative status, and drug metabolizing enzymes of lactating ewes

N. Zbib,* C. Repussard,* D. Tardieu,* N. Priymenko,† C. Domange,† and P. Guerre*2

*Université de Toulouse, INP, ENVT, UR Mycotoxicologie, F-31076 Toulouse, France, and
†Université de Toulouse, INP, ENVT, INRA UMR1331 Toxalim, F-31076 Toulouse France

ABSTRACT: Ergovaline (EV) produced by symbiotic association of Epichloë coenophiala with tall fescue (Lolium arundinaceum) causes toxicoses in livestock. In this study, 16 lactating ewes (BW 76.0 ± 0.6 kg) were used to determine the effects of feeding endophyte-infected (FE+) or endophyte free (FE−) tall fescue hay on animal health and performances and to investigate the putative mechanisms of action of EV. The mean EV concentrations in FE+ and FE− diets were 497 ± 52 and <5 µg/kg DM, respectively. Decreased hay consumption and BW were observed in the FE+ group. Prolactin (PRL) concentrations decreased (P < 0.02) in the FE+ group from d 3 to 28 of the study compared to the FE− group, but no consequences were observed on milk quantity or quality. Skin temperature and the thermocirculation index were lower (P < 0.05) in the FE+ than in the FE− group from d 3 to 7, but this effect disappeared from d 14 to 28. Hematocrit, mineral and biochemical, and enzymatic analyses of plasma revealed no differences between the 2 groups. Measurement of oxidative damage and antioxidant enzyme activities revealed a decrease in the activities of plasma catalase (P < 0.05), kidney glutathione reductase and peroxidase and in kidney total glutathione and malondialdehyde contents (P < 0.02) in ewes fed FE+. Hepatic flavin monooxygenase enzyme activities decreased (P < 0.01) in ewes fed FE+, except for a marked increase in the demethylation of erythromycin. This activity is linked to cytochrome P4503A content and is known to be involved in ergot alkaloid metabolism. Glutathione S-transferase activity in the kidneys decreased (P < 0.02) in the FE+ group, whereas no difference was observed in uridine diphosphate-glucuronosyltransferase activity in the liver or kidneys. The reversibility of the effect of FE+ hay on skin temperature and the increase in erythromycin N-demethylase activity may contribute to the relative resistance of ewes to EV toxicity.

Key words: drug metabolizing enzymes, endophyte-infected tall fescue, ergovaline, ewes, oxidative status, prolactin

INTRODUCTION

The development of Epichloë coenophiala (formerly named Neotyphodium coenophialum, Leuchtmann et al., 2014) in tall fescue (Lolium arundinaceum) leads to production of ergot alkaloids of which ergovaline (EV) is the most abundant (Tor-Agbidye et al., 2001; Repussard et al., 2013). This symbiotic association is responsible for fescue foot, summer slump syndrome, and economic loss of livestock (Schmidt and Osborn, 1993; Strickland et al., 1993; Bacon, 1995). Animal species, external temperature, and farming practices play a role in the occurrence and severity of the diseases indicated above (Spiers et al., 2005; Zbib et al., 2014).

The interaction of EV with biogenic amine receptors alters peripheral blood flow leading to hyperthermia during heat stress and to tissue necrosis when the temperatures are low (Oliver and Abney, 1989; Oliver et al., 1993; Strickland et al., 1993). Oxidative status has been reported to play a critical role in the severity of the diseases under heat stress (Lakritz et al., 2002; Spiers et al., 2005). In addition, the binding of EV to the dopaminergic receptors
of the pituitary gland inhibits prolactin (PRL) secretion (Schillo et al., 1988; Strickland et al., 1994). A decrease in PRL concentration has been observed during endophyte-infected tall fescue (FE+) exposure in all animal species investigated but its consequences are difficult to estimate (Oliver, 1997; Zbib et al., 2014). Among the hypotheses proposed to explain sensitivity to the toxin, some involve interactions with drug metabolizing enzymes. Strains of mice with high glutathione S-transferase activity are resistant to FE+ toxic effects, whereas inhibition of P4503A activity increases ergot alkaloid toxicity (Hohenboken and Blodgett, 1997; Liaudet, 1999; Wagner et al., 2000).

The purpose of this study was to investigate the impact of the consumption of FE+ hay, produced under French agricultural conditions, on animal health and milk production in lactating ewes. Additional effects on skin temperature, oxidative damage, PRL, and drug metabolizing enzymes activities were investigated to explain EV mechanisms of action in ewes.

**MATERIALS AND METHODS**

All experimental procedures involving animals were in accordance with the French National Guidelines for the care and use of animals for research purposes.

**Hay Production, Animal Exposure, and Sampling**

Studies were conducted at the agricultural school “La Cazotte” (12400 Saint-Affrique, France). Two homogeneous 0.75 ha plots were sown in 2009 in the fall with Kentucky 31 tall fescue, one with endophyte-free (FE−) seeds, and the other with FE+ seeds (infection rate of 94% on seeding) obtained from RAGT (12000 Rodez, France). The hay was harvested at maturity in June 2012 and stored in a hangar until the beginning of the assay with ewes in November 2012. One kg of hay was taken from 5 different locations of the bales after harvest and during the animal assay for chemical analysis. Dry matter was around 90%. Crude protein was 82 and 44 g/kg DM, crude fiber was 391 and 429 g/kg DM, net energy for lactation intake was 1.003 and 0.901 Mcal/kg DM for FE− and FE+, respectively. Ergovaline was determined fluorimetrically after HPLC separation as described below. Other ergot alkaloids (ergotamine, ergotaminine, ergocryptine, ergocryptinine, ergocornine, ergocorninine, ergosine, ergosinine, ergometrine, ergometrinine, ergocristine, ergocristinine) were determined by HPLC-MS (Qualttech SA laboratory, 54503 Vandoeuvre, France) and were below the limit of detection (3 µg/kg).

The ewes used in this study were housed in a large barn characterized by low stocking density and high ventilation. Italian ryegrass was used as forage before the beginning of assay with tall fescue hay. Daily, a complementary feed was supplied individually (head locks): 700 g of high-protein concentrate containing 40% of crude protein (DM basis; Evialis, 84270 Vedène, France), 500 g barley, and 20 g multivitamin supplement (Unicor, 12032 Rodez, France). Three days before the beginning of the study, 16 lactating ewes (Lacaune breed, BW 76.0 ± 0.6 kg) were selected from a herd of 48 ewes to minimize individual variations. Ewes were randomized by weight and milk production and divided into 2 homogeneous groups of 8 housed in 2 parks of 15 m² each. On d 1 that corresponded to day in milk 31 ± 4, long-stem FE− or FE+ hay was provided ad libitum for 28 d. Orts were removed daily and then around 20 kg of new hay were provided on self-service racks. Symptomatology was checked daily for signs of stress (hyperventilation), disease (prostration, diarrhea), or any abnormal comportment. Body weight, feed consumption, and milk production were recorded on d 1 and 22. Two milk samples (50 mL each) were collected on the same days. The first was analyzed for fat, protein contents, and number of cells according to the French Ministry of Agriculture for the payment of the Milk Quality (LIAL laboratory, 15000 Aurillac, France). The second was frozen at -20°C until ergovaline determination.

On d 0, 3, 7, 14, 21, and 28, temperatures were measured and blood samples were collected 1 h after milking. Core body temperature (Tco) was measured via a handheld digital thermometer placed approximately 3 cm into the rectum. Skin temperature (Ts) was determined in the medial area of the caudal side of the ear using a thermo-flash infrared thermometer. Air temperature was measured with the same apparatus. Blood samples were taken by jugular vein puncture in lithium heparinized tubes (Terumo, Leuven, Belgium). One tube was stored at room temperature for 5 h before hematocrit determination. Another was immediately centrifuged at 3,000 × g for 10 min to collect plasma. Five hundred microliters were deproteinized with metaphosphoric acid (1.25 M, vol/vol) for future determination of glutathione content. The rest of the plasma and deproteinized plasma samples were stored at -80°C until analysis.

Ewes were euthanized at d 28 by exsanguination after stunning at the Saint-Affrique slaughterhouse. Postmortem examination was conducted to reveal anomalies. The carcass, liver, kidneys, and adrenals were weighed. Fifty grams of tissues (brain, liver, kidneys, muscle, and abdominal fat) were collected, frozen in liquid nitrogen, and stored at -80°C until analysis. The animal carcasses were dispatched for rendering.

**Thermocirculation Index, Hematocrit, Biochemistry, and Prolactin Determinations**

The thermocirculation index (TCI) was calculated (Burton and Edholm, 1955) according to the following equation: TCI = (Ts − Ta)/(Tco − Ts).
Hematocrit was determined on heparinized blood in a microcapillary tube at 11,000 × g for 15 min. Plasma analytes were assayed for Na, K, urea, creatinine, total bilirubin, total protein, cholesterol, and triglycerides. For enzymes, the following specific activities were also assayed: lactate dehydrogenase (EC 1.1.1.27), aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2), and creatine kinase (EC 2.7.3.2). All parameters were performed at the Salvetat medical laboratory in Saint-Gilles (31880 La Salvetat Saint-Gilles, France) using a clinical chemistry analyzer (Hitachi 717, Tokyo, Japan) following the manufacturer’s recommendations.

Plasma was assayed for PRL by RIA following the procedure of Kann (1971). The analyses were conducted at the Neuroendocrinology Laboratory (INRA, Nouzilly, France). The sensitivity of this method is 2.5 ng/mL with intra- and interassay CVs of 7 to 12% and 10 to 14%, respectively.

**Ergovaline Determination in Hay, Tissues, and Milk**

Ergovaline standard was kindly provided by G. E. Rottinghaus (College of Veterinary Medicine, University of Missouri). All reagents and chemicals, HPLC grade, were purchased from Sigma Chemical Co., (St. Louis, MO).

**Extraction**

One kilogram of hay was dried for 24 h at 60°C and ground to 0.5 mm with a grinding mill (Cyclotech 1093, Foss, Hogande, Sweden). Five grams were extracted with alkaline chloroform (100 mL chloroform + 5 mL NaOH 0.1 M) for 2 h on an orbital shaker (Rotatest 400, Fischer Scientific, Illkirch, France) and then filtered (Whatman PS1, GE Healthcare Life Sciences, Piscataway, NJ). Ten mL of the filtered solution were then recuperated for ergovaline purification (Rottinghaus et al., 1991).

Three grams of tissues were added to 6 mL of phosphate buffer (pH 7.6, 0.1 M) and homogenized with an Ultra Turrax (TP 18, IKA, Staufen, Germany). Three mL of the suspension were collected and extracted twice with 6 mL of chloroform on an orbital shaker for 1 h. The chloroform extracts were pooled and filtered as previously described. Fat and brain were directly extracted with chloroform without homogenization. The whole amount of filtered solution was used for ergovaline purification (Durix et al., 1999).

**Purification**

Chloroform extracts were purified on homemade ergosil columns prepared as follows: 1 cm (about 150 mg) of Ergosil silica gel (Analtech Inc., Newark, DE) was placed in a 5 mL empty Solid Phase Extraction (SPE) cartridge (Silicycle, Quebec, Canada), followed by a biological disk (Silicycle, Quebec, Canada) and 1 cm of sodium sulfate (about 1,000 mg) was added. The columns were first preconditioned with 3 mL of chloroform, then the extracted samples were passed through the column. Columns were washed with 5 mL of acetone-chloroform (75:25, vol/vol) and dried for a few minutes. Ergovaline was eluted with 3 mL of methanol. The eluate was collected and evaporated to dryness with a sample concentrator at 45°C under a stream of nitrogen (Rottinghaus et al., 1991; Durix et al., 1999).

**Dosage**

The dry residue was dissolved in methanol (100 to 500 μL) and 20 to 40 μL were injected into the HPLC system comprising a pump (M 2200, Bischoff, Leonberg, Germany) connected to a C18 column (Prontosil, 250 × 4.6 mm, Bischoff, Leonberg, Germany). The mobile phase was composed of acetonitrile-water (35:65, vol/vol) with 200 mg ammonium carbonate added per liter. The flow rate was 1.5 mL/min. Fluorescence was detected (250 nm excitation and 420 nm emission) by a RF-10A XL fluorometer (Shimadzu, Japan). The resulting chromatograms were monitored by PIC 3 software (ICS, Toulouse, France). Ergovaline retention time was 9.5 min. The limits of detection were 5, 0.15, and 0.15 ng/g and ng/mL with mean recovery rates of 83, 91, and 99% in hay, tissues, and milk, respectively.

**Preparation of the Tissue Fractions and Protein Assay**

Chemicals and compounds were purchased from Sigma (St. Louis, MO). Microsomal and cytosolic fractions were prepared at 4°C. Four grams of tissue were homogenized in 11 mL 0.1 M phosphate buffer (0.1 M Tris acetate; 0.1 M KCl; 1 mM EDTA; 0.02 M butylated hydroxytoluene) at pH 7.4. The samples were then centrifuged at 9,000 × g for 30 min. The supernatant fraction was collected and centrifuged at 105,000 × g for 60 min. The upper phase (cytosolic fraction) was collected. Five hundred microliters were deproteinized with metaphosphoric acid (1.25 M, vol/vol) for future determination of glutathione content. The rest of the cytosolic fraction and the deproteinized fraction were stored at -80°C until
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1.17.3.2, 1 mU) in the presence of nitroblue tetrazolium activity was based on the inhibition of the forma-

2 min of kinetics) by monitoring the decrease in NADPH absorbance at 340 nm (Paglia and Valentine, 1967).

Total glutathione was measured using an optimized enzymatic recycling method for the quantification of GSH (Baker et al., 1990). The sulfhydryl group of GSH reacts with Ellman’s reagent 5,5′-dithio-bis-(2-nitrobenzoic acid) to produce 5-thio-2-nitrobenzoic acid (TNB) and the mixed disulfide between GSH and TNB (GS-TNB). Both GSSG and GS-TNB are reduced by GR to recycle the GSH, which produces more TNB. The rate of TNB was spectrophotometrically measured at 405 nm (50 μL of deproteinized samples in a total reaction volume of 200 μL). Dilution of the deproteinized cytosolic fraction (1/100 and 1/5 for liver and kidneys, respectiv-

ly) with 0.2 M 2-(N-Morpholino) EthaneSulfonic acid (MES) buffer (pH 6.0; 2-(N-morpholino) ethanesulfonic acid; 0.05 M K2HPO4; 1 mM EDTA) is required before determination. Exclusive of GSH, GSSG is obtained by a first derivatization of GSH with 1 M 2-vinylpyridine before measurement of total glutathione (Griffith, 1980).

Drug Metabolizing Enzyme Activities

The N-demethylation activities were measured in the microsomal fraction of tissues (1 mg protein/mL, 20 min incubation at 37°C) with aminopyrine, benzphetamine, ethylmorphine, or erythromycin (20 mM each) and N,N-dimethylnitrosamine (100 mM) as substrates. The activities were estimated through the formation of formalde-

hyde detected by the method of Nash (1953) modified by Cochin and Axelrod (1959). Formaldehyde formation was determined spectrophotometrically at 405 nm.

Dealkylation of 7-methoxyresorufin, 7-ethoxyreso-

rufin, and 7-penthoxyresorufin (2.55 mM each) were measured in the microsomal fraction of tissues (0.5 mg protein/mL, 10 min incubation at 37°C) by fluorescence (Ex 535 nm, Em 582 nm) after butanolic extraction of resorufin using the method of Lake (1987).

The uridine 5′-diphospho (UDP) -glucuronyltrans-

ferase activity in the microsomal fraction of tissues (1 mg protein/mL) with p-nitrophenol (PNP) as sub-

strate (0.7 mM) was spectrophotometrically measured (405 nm) by monitoring (12 and 3 min of kinetics for liver and kidneys, respectively) the formation of PNP-glucuronide (Frei, 1970).

Glutathione S-transferase activity (Habig et al., 1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (8 mM), was determined in the cytosolic fraction of the liver and kidneys (5 and 50 μg protein/mL, respectively) by monitoring the formation of CDNB-
conjugate (spectrophotometrically dosed at 340 nm, 2 min of kinetics). The same protocol was used with 1,2-dichloro-4-nitrobenzene as substrate (1 and 2 mg protein/ml for the liver and kidneys respectively).

Statistical Analyses

Statistical analyses were performed using R software (2.11.1, R Foundation for Statistical Computing, Vienna, Austria). Body weight, milk production, temperatures, TCI, prolactin concentration, biochemistry, and oxidative stress in plasma were compared using a linear model to assess the effect of group, time, and the interaction between group and time. When a significant effect was obtained ($P < 0.05$), a complementary range test (Wilcoxon) was used for the individual comparison of means. Oxidative stress parameters and biotransformation enzyme activities in tissues were compared using Student's test after verification of normality (Shapiro) and equality of variance (Fisher).

RESULTS

Hay and Effects on Health, Feed Intake, Body Weight, Milk Production, and Residues

Ergovaline concentrations in FE+, measured on d 0, 14, and 21, were 437, 533, and 521 µg/kg DM, respectively (mean of 3 determinations). All the analyzed samples were below the limit of detection (20 µg/kg DM) in FE− bales. No ewes died during the study, and no signs of toxicity were observed. Effects of consumption of FE− or FE+ hay on BW, milk production, and feed consumption are shown in Fig. 1. A significant decrease in BW was observed from d 1 to 22 in both groups. Because slight differences in BW were observed between the groups on d 1, individual decreases in BW were calculated (data not shown). The mean ± SD decrease in BW between d 1 to 22 was 8 ± 4 and 12 ± 3 kg in FE− and FE+, respectively. This difference was significant ($P = 0.02$), demonstrating an additive effect of the consumption of the FE+ hay on the BW decrease that occurs during lactation. By contrast, although a significant decrease in milk production was observed with time in the 2 groups ($P = 0.002$), there was no difference between FE− and FE+. The average hay consumption was less in the FE+ group than in the FE− group on d 1 and 22. Over the 28 d of the study, mean hay consumption was around 40 kg DM/ewe in the FE− group and 27 kg DM/ewe in the FE+ group. Average hay moisture (10.1%), mean FE+ consumption (30 kg/ewe), mean BW (70 kg/ewe), and mean EV concentration (497 µg/kg DM) enabled calculation of a mean intake of 6.8 µg EV/kg BW in ewes fed FE+ hay.

Analyses of milk quality were performed on d 1 and 22. No difference in fat and protein contents was observed between groups or in the number of cells (data not shown). Ergovaline residues were also measured in milk. All samples were below the limit of detection (0.15 ng/mL).

At the end of the study, postmortem examination failed to reveal any pathological alteration. No differences in the weight of the carcass, liver, kidneys, and adrenals were observed between FE− and ewes fed FE+ hay. Ergovaline residues, measured in the brain, liver, kidneys, muscle, and abdominal fat, were below the limit of detection (0.15 ng/g) in all the animals studied.

Temperatures and Thermocirculation Index

Core body temperature, skin temperature, and air temperature are listed in Fig. 2A. These data enabled the determination of a TCI, which is shown in Fig. 2B. Core body temperature remained constant throughout the study in both groups. By contrast, air temperature varied (from 19 to 5°C), leading to variations in the skin temperature and TCI in both groups. Comparison of data obtained from the FE− and FE+ groups revealed significantly lower skin temperature in FE+ ewes on d 3 and significantly lower TCI on d 3 and 7. From d 14 to 28, skin temperature and TCI did not differ in the 2 groups.

Plasma Prolactin Concentration

Plasma prolactin concentrations are shown in Fig. 3. Prolactin values decreased ($P < 0.001$) from d 0 to 28 in both groups. A linear model was used to assess intergroup variations in PRL concentrations with time. The model revealed significant differences between groups, with an effect of time ($P < 0.05$), which led us to use a complementary range test (Wilcoxon) for individual comparisons of means. In the FE− group, the decrease in the concentration of PRL from d 0 was significant on d 14 and 28 ($P < 0.05$; Fig. 3). By contrast, in the FE+
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There was a decrease ($P = 0.001$) from d 3 until d 28. When intergroup comparison was performed on each measurement day (Fig. 3), the concentrations of PRL were lower in the ewes that received the FE+ hay than in the FE−, on d 3, 7, 14, and 28.

**Biochemistry**

Hematocrit, plasma analytes, and enzymes are listed in Table 1. A decrease in sodium concentration with time was observed in both FE− and FE+ groups, whereas urea increased during the same period. In ewes fed with FE− hay, a significant increase in cholesterol and a significant decrease in triglycerides was observed between d 0 and 28, whereas these parameters remained constant in the FE+ group throughout the study period. When intergroup comparison was performed, cholesterol and triglycerides were significantly different in FE− and FE+ groups at d 28.

**Oxidative Damage and Antioxidant Enzyme Activities**

Oxidative damage and antioxidant enzyme activities were measured in plasma (Table 1) and in the liver and kidneys (Table 2). In plasma, total glutathione content (TGS) decreased with time in both groups ($P < 0.01$), with no significant difference between groups on d 28. Catalase activity also decreased with time, but the decrease was more pronounced in the ewes fed with FE+ than in control ewes fed with FE− hay. Significant decreases in MDA (cytosolic fractions) and total glutathione concentrations were observed in the kidneys of the ewes fed the FE+ in comparison to the ewes fed FE− ($P = 0.014$ and 0.001, respectively). These decreases were accompanied by a decrease in glutathione reductase and in glutathione peroxidase activities in kidneys in the FE+ group. Concerning the liver, no difference between groups was observed, whatever the parameter investigated.

**Drug Metabolizing Enzyme Activities**

The effects of 28 d of consuming FE− or FE+ tall fescue on hepatic and renal drug metabolizing enzymes are listed in Table 3. A significant decrease in ethoxyresorufin, methoxyresorufin, and pentoxyresorufin O-dealkylases activities was observed in the liver of ewes fed with FE+ hay. By contrast, no differences between groups were observed in O-dealkylases activities in kidneys. Concerning N-demethylase activity in the liver, a decrease was observed in the FE+ group when dimethylnitrosamine was used as substrate, whereas an increase was observed when erythromycin was used as substrate. No activity of N-demethylation was detected in kidneys, whatever the substrate used.

Glutathione S-transferase activity was measured in the liver and kidneys using CDNB and 1,2-Dichloro-4-NitroBenzene (DCNB) as substrates (Table 3). No difference in activity between groups was observed in the liver. By contrast, in the kidneys, a marked decrease in glutathione S-transferase activity with DCNB as substrate was observed in the FE+ group, whereas no difference was observed when CDNB was used. The uridine diphosphate-glucuronosyltransferase (UDPGT) activity, using PNP as substrate, was not affected by consuming FE+ hay, either in the liver or the kidneys.
DISCUSSION

Animal Monitoring and Production

No signs of toxicity or pathological alteration were observed during the postmortem examination in this study. For ewes fed FE+ hay for 28 d in the stable, this result is consistent with data obtained in the same species at the same physiological stage in the pasture in September. Sheep grazing tall fescue containing 458 µg EV/kg DM did not develop any signs of toxicity (Tor-Agbidye et al., 2001). This result confirms the greater resistance of sheep compared to cattle (Tor-Agbidye et al., 2001). A decrease in BW was observed in steers at a lower dose of 322 µg EV/kg DM after 21 d of exposure (Brown et al., 2008). Although room temperature in the stable was around 5°C for 7 d (d 16 to 23 of the study), this period of cold was too short to allow time for lameness to appear. Indeed, 21 d at 8°C are reported to be necessary for sheep to develop signs of toxicity at an EV concentration of 520 µg/kg DM in grass (Tor-Agbidye et al., 2001).

The consumption of hay was lower in the FE+ group that contained 497 µg EV/kg DM than in FE−. Decrease of hay consumption has been reported in cattle fed FE+ forage (Schmidt et al., 1982) and decreases in palatability in lambs and heifers were mentioned (Emile et al., 2000). Also, a decrease in the consumption of feed is common when seeds are added to feed. Such decreases have been reported in lambs, nonlactating ewes, calves, and dairy cows at a relatively high final EV level in the feed (640; 750; 1,000; and 1,400 µg/kg feed, respectively; Lakritz et al., 2002; Gadberry et al., 2003; Looper et al., 2006; Spiers et al., 2012). Both BW and milk production decreased with time in lactating ewes, which is a physiological response (Convey, 1974), but a larger decrease in BW was observed in the FE+ ewes than in FE−. This additional effect could be due to the decrease in the consumption of FE+ hay. This effect has never been reported at this dose in sheep, whereas a decrease in BW gain is common in calves (Hoveland et al., 1983; Nihsen et al., 2004; Watson et al., 2004; Burke et al., 2007; Brown et al., 2008; Spiers et al., 2012). No change in milk quality or additional decrease in milk production linked to FE+ consumption was observed in this study. Although no data is available on the effects of EV on milk production and quality in sheep, hay containing 782 µg EV/kg was reported to decrease fat and protein contents in dairy cows (Kim et al., 2007). No EV residue was detected in milk after 28 d of exposure to 6.8 µg EV kg−1 BW d−1. This result strengthens data obtained in lactating goats, demonstrating that EV excretion

Table 1. Biochemical and oxidative stress parameters in plasma of ewes fed endophyte free (FE−) or endophyte-infected (FE+) tall fescue

<table>
<thead>
<tr>
<th>Item</th>
<th>FE−</th>
<th>FE+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d 0</td>
<td>d 28</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>31.0 ± 5.1</td>
<td>31.3 ± 4.0</td>
</tr>
<tr>
<td>Sodium, mE/L</td>
<td>150 ± 3</td>
<td>144 ± 6</td>
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<tr>
<td>Potassium, mE/L</td>
<td>4.9 ± 0.6</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>Protein, g/L</td>
<td>78.6 ± 7.1</td>
<td>76.0 ± 9.9</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>6.4 ± 1.2</td>
<td>11.9 ± 2.5</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>51.7 ± 8.1</td>
<td>51.3 ± 9.2</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>0.17 ± 0.05</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.23 ± 0.49</td>
<td>1.72 ± 0.27</td>
</tr>
<tr>
<td>Total bilirubin, µmol/L</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>AST, UI/L</td>
<td>138 ± 77</td>
<td>93.5 ± 20.6</td>
</tr>
<tr>
<td>ALT, UI/L</td>
<td>12.1 ± 5.9</td>
<td>15.0 ± 4.2</td>
</tr>
<tr>
<td>CPK, UI/L</td>
<td>200 ± 111</td>
<td>152 ± 140</td>
</tr>
<tr>
<td>LDH, UI/L</td>
<td>611 ± 91</td>
<td>459 ± 83</td>
</tr>
<tr>
<td>MDA, nmol/mg</td>
<td>9.5 ± 2.5</td>
<td>10.5 ± 2.3</td>
</tr>
<tr>
<td>SOD, µU min−1 mg−1</td>
<td>2.28 ± 0.32</td>
<td>2.32 ± 0.28</td>
</tr>
<tr>
<td>CAT, ng CH₃O min−1 mg−1</td>
<td>88.2 ± 14.9</td>
<td>67.2 ± 19.0</td>
</tr>
<tr>
<td>GR, µmol NADPH min−1 mg−1</td>
<td>0.33 ± 0.08</td>
<td>0.28 ± 0.11</td>
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<tr>
<td>GPx, µmol NADPH min−1 mg−1</td>
<td>0.82 ± 0.04</td>
<td>0.80 ± 0.19</td>
</tr>
<tr>
<td>TGSH, pmol/mg</td>
<td>14.3 ± 7.6</td>
<td>8.35 ± 3.36</td>
</tr>
</tbody>
</table>

*Significant difference from d 0.

**Significant difference between groups at the same day (Wilcoxon, P < 0.05).

1 Values are expressed as mean ± SD, n = 8.

2 AST = aspartate aminotransferase; ALT = alanine aminotransferase; CPK = creatine phosphokinase; LDH = lactate dehydrogenase; MDA = malondialdehyde; SOD = superoxide dismutase; CAT = catalase; GR = glutathione reductase; GPx = glutathione peroxidase; TGSH = total glutathione; CH₂O = formaldehyde; NADPH = nicotinamide adenine dinucleotide phosphate.

3 mg of plasma proteins.
in milk is very low after an IV route, only 0.05% of the administered dose (Durix et al., 1999). It also corroborates the lack of EV detection in milk after intraruminal administration of a single dose of EV (38 µg EV/kg BW) to lactating goats (Grancher, 2007).

### Temperatures and Thermocirculation Index

In this study, core body temperature remained constant, suggesting that a balance between heat production and heat loss was maintained. The TCI was developed to identify vasomotor effect of EV and other ergot alkaloids, which are considered to be responsible for the signs of lameness and necrosis that appear during fescue toxicosis (Burton and Edholm, 1955; Oliver et al., 1993). The TCI has been reported to decrease in lambs after 14 d of feeding a diet containing 640 µg EV/kg (Gadberry et al., 2003). In this study, a decrease in TCI was obtained on d 3 and 7. This effect was linked to a decrease in skin temperature, measured on the ear. It demonstrated the rapid effect of EV on peripheral blood flow, in agreement with data obtained in rats where a decrease in external temperature was observed 15 min after intraperitoneal injection (Spiers et al., 1995). A rapid reduction in tail skin temperature (30 min) was also obtained in cattle after IV dosing (McCollough et al., 1994). Interestingly, after 14 d of exposure, no difference was observed between FE− and FE+ fed ewes, even with an external temperature of 5°C, suggesting that the effects of EV on peripheral blood flow in lactating ewes are brief. The disruption of basic homeostatic processes plays a key role in potentially vulnerability to the effects of endophyte-infected fescues. Animal age and species are known to be determining factors in thermoregulatory response to FE+ toxins, especially under heat stress (Spiers et al., 1995, 2005, 2012). However, although several studies have been conducted in cattle and rats, little data is available in sheep (Spiers et al., 2005). This result is nevertheless of interest with respect to the proposed mechanism of action of EV. Reduced blood

### Table 2. Effects of 28 d of feeding ewes endophyte-free (FE−) or endophyte-infected (FE+) tall fescue on oxidative stress parameters

<table>
<thead>
<tr>
<th>Item</th>
<th>d 28 FE−</th>
<th>d 28 FE+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver, g</td>
<td>995 ± 183</td>
<td>894 ± 148</td>
</tr>
<tr>
<td>MDA, nmol/mg of cytosolic prot</td>
<td>63.4 ± 14.3</td>
<td>76.8 ± 12.2</td>
</tr>
<tr>
<td>MDA, mmol/mg of microsomal prot</td>
<td>6.35 ± 4.31</td>
<td>5.34 ± 0.68</td>
</tr>
<tr>
<td>SOD, U min−1 mg−1 of cytosolic prot</td>
<td>27.0 ± 8.9</td>
<td>22.8 ± 6.1</td>
</tr>
<tr>
<td>CAT, µg CH2O min−1 mg−1 of cytosolic prot</td>
<td>376 ± 119</td>
<td>327 ± 72</td>
</tr>
<tr>
<td>GR, µmol NADPH min−1 mg−1 of cytosolic prot</td>
<td>30.6 ± 7.4</td>
<td>30.4 ± 6.8</td>
</tr>
<tr>
<td>GPx, µmol NADPH min−1 mg−1 of cytosolic prot</td>
<td>120 ± 23</td>
<td>102 ± 15</td>
</tr>
<tr>
<td>TSH, µmol of cytosolic prot</td>
<td>36.8 ± 16.9</td>
<td>21.2 ± 12.8</td>
</tr>
<tr>
<td>GSSG, µmol of cytosolic prot</td>
<td>6.15 ± 2.93</td>
<td>3.09 ± 1.50</td>
</tr>
<tr>
<td>Kidneys, g</td>
<td>193 ± 40</td>
<td>171 ± 31</td>
</tr>
<tr>
<td>MDA, nmol/mg of cytosolic prot</td>
<td>69.7 ± 11.5</td>
<td>53.6 ± 7.7</td>
</tr>
<tr>
<td>MDA, mmol/mg of microsomal prot</td>
<td>22.0 ± 4.9</td>
<td>25.0 ± 2.5</td>
</tr>
<tr>
<td>SOD, U min−1 mg−1 of cytosolic prot</td>
<td>8.59 ± 0.93</td>
<td>9.98 ± 2.68</td>
</tr>
<tr>
<td>CAT, µg CH2O min−1 mg−1 of cytosolic prot</td>
<td>57.1 ± 14.4</td>
<td>54.9 ± 15.9</td>
</tr>
<tr>
<td>GR, µmol NADPH min−1 mg−1 of cytosolic prot</td>
<td>48.2 ± 6.4</td>
<td>38.0 ± 4.2</td>
</tr>
<tr>
<td>GPx, µmol NADPH min−1 mg−1 of cytosolic prot</td>
<td>83.3 ± 10.6</td>
<td>71.6 ± 8.7</td>
</tr>
<tr>
<td>TGSH, µmol of cytosolic prot</td>
<td>2.60 ± 0.47</td>
<td>1.75 ± 0.43</td>
</tr>
<tr>
<td>GSSG, µmol of cytosolic prot</td>
<td>0.09 ± 0.06</td>
<td>0.07 ± 0.04</td>
</tr>
</tbody>
</table>

*Significant difference between groups (Wilcoxon, P < 0.05).

### Table 3. Effects of 28 d of feeding ewes endophyte-free (FE−) or endophyte-infected (FE+) tall fescue on hepatic and renal drug metabolizing enzymes

<table>
<thead>
<tr>
<th>Item</th>
<th>d 28 FE−</th>
<th>d 28 FE+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver, g</td>
<td>995 ± 183</td>
<td>894 ± 148</td>
</tr>
<tr>
<td>O-dealkylation</td>
<td>7.97 ± 1.42</td>
<td>3.66 ± 0.74</td>
</tr>
<tr>
<td>Ethoxyresorufin</td>
<td>1.67 ± 0.25</td>
<td>1.32 ± 0.17</td>
</tr>
<tr>
<td>Methoxyresorufin</td>
<td>0.58 ± 0.09</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>Pentoxresorufin</td>
<td>53.1 ± 17.5</td>
<td>47.2 ± 28.0</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>156 ± 22</td>
<td>139 ± 36</td>
</tr>
<tr>
<td>Benzphetamine</td>
<td>162 ± 27</td>
<td>146 ± 34</td>
</tr>
<tr>
<td>Dimethylaminopamine</td>
<td>85.3 ± 15.2</td>
<td>55.7 ± 21.3</td>
</tr>
<tr>
<td>Ethylnitrosamine</td>
<td>19.4 ± 11.3</td>
<td>58.6 ± 23.9</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>193 ± 40</td>
<td>171 ± 31</td>
</tr>
<tr>
<td>Ethylmorphine</td>
<td>0.81 ± 0.21</td>
<td>0.90 ± 0.20</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>1.06 ± 0.30</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>CDNB6</td>
<td>0.22 ± 0.04</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>DCNB5</td>
<td>0.12 ± 0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UDPGT6</td>
<td>0.12 ± 0.04</td>
<td>0.11 ± 0.04</td>
</tr>
</tbody>
</table>

*Significant difference between groups (Wilcoxon, P < 0.05).
flow has been demonstrated in all species in which it has been studied, and a prolonged effect has been considered necessary to induce anoxia of extremities leading to lameness followed by necrosis (Strickland et al., 1993; Oliver, 1997; Spiers et al., 2005). In this study, ewes fed the FE+ hay only showed lower skin temperature values than those fed the FE− hay at the beginning of the exposure. This agrees with all the available data on sheep, which appear to be more resistant to necrosis of the extremities than cattle (Tor-Agbidye et al., 2001).

**Plasma Prolactin Concentration**

In this study, PRL concentrations decreased with time, which is common during lactation (Convey, 1974). The decrease in PRL was more pronounced in ewes fed the FE+ hay than in the FE− hay, in agreement with several studies on the subject. A decrease in PRL concentration was observed during FE+ exposure in all the animal species investigated (Zbib et al., 2014). This effect has been linked to the direct action of EV on dopaminergic receptors of the pituitary cells (Schillo et al., 1988; Strickland et al., 1994). Ergovaline blocks the liberation of PRL through fixation on the dopaminergic receptors in the cells (Siegel et al., 1989). The decrease in PRL occurs rapidly, as observed in cattle 30 min after an IV dose of EV (McCollough et al., 1994). Because the concentration of PRL varies with the species, age, and physiological stage, the interstudies comparison is difficult. In steers, a significant decrease in PRL was observed 7 d after exposure to forage containing 317 µg EV/kg DM (Stamm et al., 1994). In lambs, a decrease in PRL was observed 14 d after exposure to feed containing 640 µg EV/kg DM or 28 d after 610 µg/kg (Gadberry et al., 2003; De Lorme et al., 2007). In ewes, a marked decrease in PRL (>90%) was observed after 42 d of exposure to a grass containing 460 µg EV/kg DM and a smaller decrease (75%) was observed after 7 d of exposure to a feed containing 750 µg EV/kg DM, suggesting that the length of exposure determines the severity of the effect (Tor-Agbidye, 1993; Tor-Agbidye et al., 2001; Looper et al., 2006). In the present study, a decrease of 60 and 73% in PRL was observed on d 3 and 28, respectively, in ewes fed with FE+, confirming the effect of time on this parameter. This result also shows that although PRL is a very sensitive biomarker of exposure to FE+ during lactation, the decrease in PRL concentration has no significant effect on milk production.

**Biochemistry**

Only a few biochemical effects were observed in this study, most of which were linked to lactation and not to the consequence of FE+ feeding. This result is in agreement with all available data on sheep, demonstrating the lack of effect of feed containing endophyte fescue (seeds or grass) on plasma analytes or enzyme biochemistry (Zbib et al., 2014). On d 28, the only differences between groups observed concerned cholesterol and triglycerides, with a decrease in cholesterol and an increase in triglycerides. A decrease in cholesterol was also reported in steers and heifers fed FE+ grass, but the decrease was accompanied by a decrease in triglycerides (Oliver et al., 2000; Nihsen et al., 2004; Brown et al., 2008), which was not the case in the present study. The effects on cholesterol and triglycerides are thus difficult to interpret; they appeared to be linked to time in the FE− fed ewes (significant difference between d 0 and 28) but not in the FE+ fed ewes (no significant difference between d 0 and 28).

**Oxidative Damage and Antioxidant Enzyme Activities**

Oxidative damage is recognized as an important mechanism that may occur during exposure to FE+ under heat stress in cattle and rodents (Lakritz et al., 2002; Spiers et al., 2005; Bhusari et al., 2006; Burke et al., 2007; Settivari et al., 2009). Most of the results obtained demonstrated a decrease in antioxidant defense mechanisms (measured by a decrease in GSH content and in antioxidant enzyme activities) and sometimes an increase in oxidative damage (measured by increased MDA and GSSG contents; Lakritz et al., 2002). In this study, only weak effects of FE+ on oxidative damage and antioxidant enzyme activities were observed. A decrease in catalase activity in plasma was observed in ewes fed FE+ forage, in agreement with data obtained in rats (Settivari et al., 2008). Slight decreases in SOD, CAT, GPx, and TGSH were also observed in the liver of FE+ fed ewes, but these effects were not significant, in contrast to data obtained in rodents under high EV exposure (Bhusari et al., 2006; Settivari et al., 2008, 2009). The GR and GPx activities decreased in the kidney of FE+ ewes, whereas a decrease in TGSH and MDA contents were also observed. Taken together, these results suggest that a moderate decrease in the mechanism of defense against oxidative damage is possible in ewes fed FE+ forage, with no significant consequences for health, as measured by biochemistry, and milk production.

**Drug Metabolizing Enzyme Activities**

Drug metabolizing enzymes are involved in the metabolism of drugs and toxic compounds. These activities can be decreased or increased by several mechanisms, with possible consequences for toxicity and for the excretion rate of chemical compounds. Previous studies conducted to explain differences between breeds or lines to fescue toxicity hypothesized that some of the observed differences are linked to differences in expression or activities of drug metabolizing enzymes. In mice, resistant lines displayed
Effects of ergovaline on lactating ewes

significantly greater Glutathione S-transferase activity than sensitive lines, whereas total cytochrome P450 and UDPGT activities were not affected (Hohenboken and Blodgett, 1997; Wagner et al., 2000). Genomic analysis of the impact of fescue toxicosis on the expression of drug metabolizing enzymes during heat stress in mice also showed that some P450 genes (Cyp2d26 and Cyp2a12) were upregulated whereas others were downregulated (Cyp3a25; Bhursari et al., 2006). Studies in rats revealed upregulation of Cyp (2c13 and 2e1) and downregulation of glutathione S-transferase genes (Settivari et al., 2006). Both direct effects on gene expression and indirect mechanisms linked to the effects of ergot alkaloids on the mechanism of defense against oxidative damages in liver have also been cited to explain the pathogenesis of fescue toxicosis (Settivari et al., 2008, 2009). In the present study, drug metabolizing enzyme activities of P450 enzymes in the liver generally decreased, with the exception of erythromycin N-demethylase activity, which increased. Erythromycin is a substrate of subfamily P450 3A, which is involved in the metabolism of ergot alkaloids, its inhibition leading to toxicity (Liaudet, 1999). Species and gender differences have been reported in in vitro metabolism of ergotamine, an alkaloid similar to EV, and it has been suggested that these differences could explain variations in sensitivity to fescue toxicosis (Duringer et al., 2005; Moubarak et al., 2006). Breed differences have also been cited to explain the differences in the prevalence of fescue toxicosis around the world. Indeed, the prevalence of the disease is low in Europe and high in the United States, Australia, and New Zealand, even though wild endophytes that produce ergot alkaloids are present in Europe (Repussard et al., 2013). Interestingly, breed differences in erythromycin N-demethylase activities have been demonstrated in cattle (Dacasto et al., 2005). Finally, because erythromycin is a substrate of P450 3A, which is involved in the metabolism of ergot alkaloids, we hypothesize that the 3-fold increase in erythromycin N-demethylase activity observed in this study in FE+ fed ewes contributes to a protective effect against EV toxicity.

By contrast, dimethylnitrosamine N-demethylase activity, which is linked to family P450 2 (Yamazaki et al., 1992; Stiborová et al., 1996; Chowdhury et al., 2012) and ethoxyresorufin, methoxyresorufin, and pentoxysresorufin N-dealkylase activities, which are linked to subfamilies P450 1A and 2B (Machala et al., 2003; Sztókáková et al., 2004), decreased, whereas P450 1A, 2B, and 2 were not reported to be involved in the metabolism of ergot alkaloids.

Glutathione S-transferase activity with DCNB as substrate decreased in the kidney but not in the liver. No data is available concerning the effect of ergot alkaloids on drug metabolizing enzymes in kidney; however, a decrease in the expression of this enzyme has been reported in mouse liver (Settivari et al., 2006). Activities of UDPGT were not affected in the liver and kidney in the present study, in agreement with previous data in rodents (Hohenboken and Blodgett, 1997; Wagner et al., 2000).

In conclusion, feeding FE+ hay containing 497 µg EV/kg DM to lactating ewes for a period of 28 d led to a decrease in consumption and a decrease in BW but no effect on milk production or quality and no residues of EV were detected. A decrease in ear temperature was detected the first week of the study, then disappeared, suggesting an adaptive response. By contrast, a decrease in PRL was observed from d 3 to 28, confirming it is a sensitive biomarker of FE+ exposure. The measurement of oxidative damage and antioxidant enzyme activities suggested a moderate decrease in the defense mechanism against oxidative damage in FE+ fed ewes, with no consequences for health in the breeding conditions used in the present study. Analysis of drug metabolizing enzyme activities revealed a general decrease in activity in ewes fed FE+ forage, with the exception of a marked increase in demethylation of erythromycin that was attributed to P450 3A activity, which is involved in the metabolism of EV.

LITERATURE CITED


Effects of ergovaline on lactating ewes


