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Measurement of urinary concentrations of the mycotoxins zearalenone and sterigmatocystin as biomarkers of exposure in mares

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Contents

Mycotoxins may affect animal health, including reproduction. Little is known about the clinical relevance of exposure of horses to contaminated feed. This study aimed at (i) monitoring the levels of the mycotoxins zearalenone (ZEN), with its metabolites α- and β-zearalenol (α- and β-ZOL), and sterigmatocystin (STC) in urine samples from thoroughbred mares in Japan and (ii) relating these findings to the potential effects on reproductive efficacy of breeding mares. Sixty-three urine samples of breeding mares from 59 breeding farms were used. Urine samples and reproductive records were collected from each mare when it was presented to the stallion station. Urinary concentrations of ZEN, α- and β-ZOL, and STC were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). ZEN, α- and β-ZOL were measurable in the urine of all examined mares, indicating the prevalence of ZEN in equine feeds. In seven of the 63 samples, STC was also detected at levels ranging from 1.3 to 18.0 pg/mg creatinine. No significant correlation between the concentrations of mycotoxins and pregnancy status was observed. In conclusion, measurement of mycotoxins in urine samples is a useful non-invasive method for monitoring the systemic exposure of mares to multiple mycotoxins.

1 | INTRODUCTION

Mycotoxins produced by the genera *Fusarium*, *Penicillium* and *Aspergillus* are known to be frequently present in horse feeds and may reduce animal health, performance and reproduction (Buckley, Creighton, & Fogarty, 2007; Caloni & Cortinovis, 2010; Raymond, Smith, & Swamy, 2003, 2005). While numerous reports describe the adverse effects of mycotoxins in common farm animals, information from horses is still limited (Fink-Gremmels & Spronck, 2013). The mycotoxin zearalenone (ZEN) has gained particular attention owing to its unique oestrogenic properties. ZEN is produced by *Fusarium* species in plants, particularly maize and small grains, rice and pasture grasses predominantly at the re-harvest stage (Dänicke & Winkler, 2015; Fink-Gremmels & Malekinejad, 2007). The ZEN lactone ring resembles many of the stereo-chemical features of the steroidal (estradiol) structure and therefore fits into the binding pocket of the mammalian oestrogen receptor. In turn, ZEN and its metabolites exhibit distinct oestrogen-mimicking properties that affect the reproductive system (Fink-Gremmels & Malekinejad, 2007; Kleinova, Zollner, Kahlbacher, Hochsteiner, & Lindner, 2002; Minervini & Dell'Aquila, 2008). While this mechanism has been described in all animal species investigated, very few in vivo trials (Juhász et al., 2001) or in vitro assays (Filannino et al., 2011; Minervini et al., 2006, 2010) have been conducted with ZEN or its metabolites in female horses. The rate of conversion to

α-zearalenol (α-ZOL) is of major importance as this metabolite has much higher oestrogenic activity and hence largely determines the clinical relevance of exposure to ZEN-contaminated feed (EFSA 2016).

Sterigmatocystin (STC) is produced by fungi of the genera *Aspergillus* and *Penicillium*. STC is the product of a biosynthetic pathway in some fungal species such as *A. versicolor* and *A. nidulans* and is a well-known precursor of aflatoxin B1 biosynthesis in other fungal species (Hsieh, Lin, & Yao, 1973; Versilovskis & de Saeger, 2010; Wilkinson, Ramaswamy, Sim, & Keller, 2004). STC is frequently reported as a contaminant in feeds (e.g., grains, maize and rice straw) at the post-harvest stage, but tolerable maximum exposure limits have not been established, and details on its adverse health effects in livestock, including horses, are scarce (EFSA 2014).

One method of estimating the levels of exposure of animals to mycotoxins is analysing feed materials of total mixed rations. Another approach is to measure toxin concentrations in biological samples such as urine, which reflects the internal dose and individual exposure levels. This method is increasingly used to measure human exposure to mycotoxins, particularly with complex mixtures of mycotoxins and their metabolites (Heyndrickx et al., 2015).

Recently, we established a novel monitoring system for ZEN and its metabolites, and STC exposure using urinary analyses in dairy herds (Fushimi et al., 2014; Takagi et al., 2011). Considering the likely exposure of horses to mycotoxin-contaminated feed materials or bedding (straw) (Séguin et al., 2012; Wichert et al., 2008), we aimed at establishing a similar monitoring system for horses. Here, we describe the analysis of ZEN, its metabolites (α-ZOL and β-ZOL) and STC in the urine of breeding mares, using a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method.

2 | MATERIALS AND METHODS

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan).

2.1 | Chemicals and solvents

Zearalenone was purchased from MP Biomedicals (Heidelberg, Germany). The metabolites α-ZOL and β-ZOL were purchased from Sigma (St. Louis, MO, USA). Methanolic stock solutions with ZEN, α-ZOL and β-ZOL concentrations of 1 μg/ml each were stored under light protection at 4°C. Sterigmatocystin (STC) was purchased from MP Biomedicals (Heidelberg, Germany). Stock solutions of 1 μg/ml STC in acetonitrile were stored in the dark at 4°C. Ammonium acetate and high-performance liquid chromatography (HPLC)-grade methanol were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). β-glucuronidase (approximately 30 U/ml)/arylsulfatase solution (approximately 60 U/ml), used during sample preparation to cleave off glucuronides and sulphate esters prior to LC-MS/MS, was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). TRIS was purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

2.2 | Urine sample collection

Sixty-three urine samples were collected from female thoroughbred horses (3-23 years old) between March and May 2014, at the stallion breeding stations in the Hidaka area of Hokkaido, Japan. Briefly, the urine samples were collected at either 10:00 a.m. or 3:00 p.m., when the mares were bred with stallions, using a urine-collection vessel (150 ml) with an extended rod. Immediately after collection, the collected urine was divided into Falcon tubes (50 ml). All samples were immediately placed in an icebox to keep cool and protected from light while being transported to the laboratory. Urine samples were centrifuged at 500 × *g* for 10 min at room temperature (20-25°C) to remove debris such as epithelial cells, and then frozen at -30°C until analysis.

Preliminary trials for extraction methods were conducted to confirm the appropriate quantification of ZEN, α-ZOL, β-ZOL and STC in horse urine, including direct extraction from urine samples and pre-incubation of the urine samples with β-glucuronidase/arylsulfatase.

2.3 | Urinary metabolites of ZEN, α-ZOL, β-ZOL and STC

All urine samples were analysed using LC-MS/MS according to our previous study (Fushimi et al., 2014; Takagi et al., 2011). Briefly, 0.5 ml of a urine sample was mixed with 3.0 ml of 50 mM ammonium acetate buffer (pH 4.8) and 10 μl of glucuronidase/arylsulfatase solution and incubated for 12 hr at 38°C. After the incubation, the solution (3.5 ml in total) was loaded onto a C18 SPE column, which was preconditioned with 3 ml of 100% MeOH and 2 ml of Tris buffer, followed by the addition of 2 ml Tris buffer and 3 ml of 40% MeOH. ZEN, α-ZOL, β-ZOL and STC were eluted from the SPE column by passing 1 ml of 80% MeOH that was collected in a vial and its volume was adjusted to 1 ml with 80% MeOH. Then, 20 μl of the reconstituted solution was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system as below.

Analyses with LC-ESI/MS/MS were performed on an API 2000 LC/MS/MS system (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization interface and an HPLC system (1200 Series; Agilent Technologies, Santa Clara, CA). The measurements were separately performed in ESI, for ZEN and its metabolites in negative mode, and STC in positive mode.

Electrospray conditions for ZEN and α/β-ZOL were as follows: curtain gas, 20 psi; ion-spray voltage, -4500 V; turbo temperature, 500°C; collision energy, -48.0 eV for ZEN and -44.0 eV for α/β-ZOL; declustering potential, -36.0 V for ZEN and -51.0 V for α/β-ZOL; focusing potential, -260 V for ZEN and -280 V for α/β-ZOL; entrance potential, -9.0 V for ZEN and -8.5 V for α/β-ZOL. Additionally, the electrospray conditions for the measurement of STC were as follows: curtain gas, 40 psi; ion-spray voltage, 5500 V; turbo temperature, 500°C; collision energy, 16.0 eV; declustering potential, 6.0 V; focusing potential, 360 V; and entrance potential, 10.5 V. Nitrogen

was used as a nebulizer, curtain and collision gas. Chromatographic separations between ZEN and its metabolites or STC were achieved on an Inertsil ODS-3 (4.6 ID × 150 mm, 5 μm; GL Sciences, Tokyo, Japan) at 40°C. The mobile phase for the measurements of ZEN and its metabolites consisted of methanol (A) and water (B), and the gradient elution was applied to separate the analyte of ZEN and its metabolites as follows: solvent A was increased from 50% to 100% in 5 min along a linear gradient curve, and the isocratic elution was held for 10 min at a flow rate of 200 ml/min. Re-equilibration of the column was isocratically performed with 50% of solvent B for 7 min at a flow rate of 1000 ml/min. The mobile phase, which consisted of methanol/water/acetic acid (97:3:0.01, v/v/v), was applied (200 μl/min) to separate the analyte in the isocratic mode. The injected sample volumes for both measurements were individually 20 μl. For the LC-MS/MS analysis, a multiple reaction monitoring system was used for the transition of ZEN (*m/z* 317.0-130.5) and α/β-ZOL (*m/z* 319.0-129.9) in a negative ion mode and STC (*m/z* 325.0-281.0) in a positive ion mode. Instrumental parameters were optimized for each analyte by analysis of the corresponding standard solution (1.0 mg/L in methanol) at a flow rate of 10 μl/min, using a syringe pump integrated in the API-2000 mass spectrometer. In this study, the recovery rate for both ZEN and its metabolites and STC were preliminarily examined using horse urine. Briefly, the urine samples from three different mares and supplemented with 100 ng/L and 10 ng/L of ZEN, α-ZOL, β-ZOL and STC were used for the recovery tests. The mean recovery rates of ZEN, α-ZOL, β-ZOL and STC were 94.3%, 84.9%, 93.2% and 73.4%, respectively, in the case of supplementation with 100 ng/L, and 76.8%, 99.8%, 81.6% and 70.2%, respectively, in the case of supplementation with 10 ng/L. Analysis of the results of ZEN and its metabolites and STC did not correct for the recovery rates.

Creatinine concentrations in the urine were determined using a commercial kit, the Sikarikit-S CRE (Kanto Chemical, Tokyo, Japan) following the manufacturer's instructions, and measured using the BM8060 Clinical Analyzer (Nihon-Denshi, Tokyo, Japan). The urinary concentrations of ZEN, α-ZOL, β-ZOL and STC were expressed as a ratio to creatinine (pg/mg Crea), as described previously (Fushimi et al., 2014; Takagi et al., 2011).

2.4 | Mating and control of the pregnancy status of mares

Pregnancy testing of each mare was conducted after mating, and non-pregnant mares were re-mated in consecutive oestrous cycles until they conceived. Mares that failed to conceive were dispatched from the breeding programme at the end of the season. Complete reproductive histories of the examined mares were recorded. The relationship between urinary mycotoxin concentrations and conception rates of the mares was evaluated using two methods based on the number of matings necessary for conception. First, mares were divided into two groups depending on whether they became pregnant at the first mating or not (non-pregnant). Second, the percentage of mares that conceived after the first breeding was compared with the number of mares that remained non-pregnant after three or four breeding attempts.

2.5 | Statistical analysis

The data on urinary ZEN, α-ZOL, β-ZOL, total ZEN (ΣZEN; ZEN + α-ZOL + β-ZOL) and STC concentrations (pg/mg Crea) were expressed as mean ± SEM. The urinary ZEN concentrations, with or without β-glucuronidase/arylsulfatase treatment during the extraction, were compared using the Wilcoxon signed-ranks test. The pregnancy status, urinary concentrations of ZEN, α-ZOL, β-ZOL, ΣZEN and STC were compared between pregnant and non-pregnant mares using the Student's *t* test with Welch correction when variances were heterogeneous. *p* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Urinary metabolites of ZEN, α-ZOL, β-ZOL and STC

The levels of ZEN, α-ZOL, β-ZOL and STC for six urine samples, with or without pre-incubation with β-glucuronidase/arylsulfatase, are presented in Table 1. Without enzymatic pre-incubation, only the free amounts of ZEN (mean 0.93 pg/mg Crea) were measurable in urine. When the samples were pre-incubated with β-glucuronidase/arylsulfatase, the levels of ZEN significantly ($p < .05$) increased (mean 1.81 pg/mg Crea), indicating that mares can excrete both the conjugated and unconjugated forms of ZEN in urine. The ZEN metabolites, α-ZOL and β-ZOL, were only measurable in their conjugated form, that is after enzymatic hydrolysis. Additionally, the mean ratio of ZEN:ΣZEN (ZEN + α-ZOL + β-ZOL) was 0.5:1, and the mean ratio of β-ZOL:α-ZOL was 1.6:1 (Table 1). STC was found in its conjugated form in only one sample from the first experiment. Based on these preliminary results, all future analyses of the urine samples were pre-incubated with β-glucuronidase/arylsulfatase prior to extraction.

3.2 | ZEN, α-ZOL, β-ZOL and STC concentrations in urine sample of examined mares

3.2.1 | Evaluation 1

The number of mares that became pregnant at the first breeding was 23 (mean 11.1 ± 1.1 years old), while 40 mares (mean 11.3 ± 0.7 years old) did not. Table 2 shows the mycotoxin concentrations (pg/mg Crea) in pregnant and non-pregnant mares, which were not significantly different.

3.2.2 | Evaluation 2

No significant differences ($p = .14$) were observed in the mycotoxin levels between pregnant ($n = 23$) and non-pregnant mares ($n = 7$; 10.3 ± 1.9 years old) that mated more than three times (Table 2).

TABLE 1 Results of urinalyses in mares, the effects of pre-incubation of urine samples with β-glucuronidase/arylsulfatase on measurable urinary concentrations (Mean ± SEM), and the ratio of zearalenone and its metabolites in urine samples

	Urinary concentrations (ng/ml)					Ratio	
	ZEN	α-ZOL	β-ZOL	ΣZEN	STC	ZEN:ΣZEN	β-ZOL:α-ZOL
Without pre-incubation							
Mare 1	0.82	nd	nd		nd		
Mare 2	0.96	nd	nd		nd		
Mare 3	0.9	nd	nd		nd		
Mare 4	0.92	nd	nd		nd		
Mare 5	1.02	nd	nd		nd		
Mare 6	0.96	nd	nd		nd		
Mean	0.93* ± 0.03						
With pre-incubation[a]							
Mare 1	1.36	0.48	0.84	2.68	nd	0.5:1	1.8:1
Mare 2	1.16	0.16	0.28	1.6	nd	0.7:1	1.8:1
Mare 3	3.44	1.18	1.56	6.18	nd	0.6:1	1.3:1
Mare 4	0.84	0.06	0.16	1.06	nd	0.8:1	2.7:1
Mare 5	1.14	0.48	0.62	2.24	nd	0.5:1	1.3:1
Mare 6	2.94	2.0	1.4	6.34	0.24	0.5:1	0.7:1
Mean	1.81* ± 0.44					0.5: 1	1.6: 1

ZEN, α-ZOL and β-ZOL: LOQ; 0.05 ng/ml, LOD; 0.02 ng/ml.
STC: LOQ; 0.1 ng/ml, LOD; 0.05 ng/ml.
[a]pre-incubated with β-glucuronidase/arylsulfatase mixture.
nd: not determined.
*Significant difference ($p < .05$).

While there was generally one mare sampled per farm, samples were obtained from two mares at each four farms. Their results were analysed as pairs to correct for this unequal sampling. The paired ΣZEN concentrations (pg/mg Crea) were as follows: farm 1, 193.4 and 119.4; farm 2, 136.9 and 52.4; farm 3, 132.6 and 83.2; and farm 4, 140.2 and 113.0. Paired concentrations of ΣZEN (pg/mg Crea) within the same farm were almost equal.

Seven of the 65 examined mares showed a clear peak of STC when analysed by LC-MS/MS, with concentrations ranging from 1.3 to 18.0 (pg/mg Crea). There was no significant relationship between STC exposure and pregnancy status of mares.

4 | DISCUSSION

Mycotoxin exposure can be measured by feed analysis, but this provides limited information on the internal dose that is responsible for most of the adverse effects of mycotoxins in animals. As the exact rates of absorption and biotransformation of mycotoxins are known for a variety of farm animals, including horses (Fink-Gremmels & Spronck, 2013; Malekinejad, Maas-Bakker, & Fink-Gremmels, 2006; Songsermsakul, Böhm, Aurich, Zentek, & Razzazi-Fazeli, 2013), analysis of urine samples could provide additional information on the internal dose (Songsermsakul et al.,

TABLE 2 Comparison of mean urinary concentrations (mean ± SEM; pg/mg Crea) of individual mycotoxins between pregnant (Preg) and non-pregnant (Non-preg) mares

	ZEN	α-ZOL	β-ZOL	ΣZEN	STC[a]
Evaluation 1					
Preg (n = 23)	55.7 ± 10.1	48.1 ± 11.2	67.6 ± 16.0	161.4 ± 32.0	5.6–18.0 (n = 2)
Non-preg (n = 40)	78.5 ± 12.0	59.0 ± 9.0	64.3 ± 9.6	196.8 ± 28.0	1.3–5.5 (n = 5)
Evaluation 2					
Preg (n = 23)	55.7 ± 10.1	48.1 ± 11.2	67.6 ± 16.0	161.4 ± 32.0	5.6–18.0 (n = 2)
Non-preg (n = 7)	96.5 ± 37.5	60.3 ± 15.5	72.0 ± 29.6	228.8 ± 80.9	3.5–5.5 (n = 2)

No statistical differences were found between pregnant and non-pregnant mares.
Evaluation 1, comparison of non-pregnant mares after the first breeding attempt; Evaluation 2, comparison with total failure to conceive in the season.
[a]indicates the concentration range and number of positive samples.

2013). Urine sampling is preferred over other biological materials, such as blood samples, as it is non-invasive and reflects the entire elimination phase, including the excretion of so-called modified mycotoxins. Modified mycotoxins, previously denoted as masked mycotoxins, are plant (glucose) conjugates that escape common extraction methods applied in feed analyses. In addition, modified mycotoxins can also be produced by toxigenic fungi (Berthiller et al., 2013). The intestinal microbiota has the ability to cleave glycosidic bonds, and the liberated parent toxins contribute to the overall animal exposure (Berthiller et al., 2013; EFSA, 2014; Kovalsky et al., 2014). Moreover, urine sampling allows the determination of all main metabolites.

One of the main objectives of this study was to investigate the suitability of a previously described analytical method, originally developed for cattle, for monitoring mycotoxin exposure in horses. To our knowledge, this is also the first study to describe the contamination status of ZEN, its metabolites and STC in horses. Based on the mean ratio of ZEN:ΣZEN (0.5:1), ZEN appears to be the main metabolite in the urine of horses. Interestingly, these results also indicate that almost 100% of α- and β-ZOL are excreted as glucuronide conjugates, with a mean ratio of β-ZOL:α-ZOL of 1.6:1. However, some amount of ZEN (0.82–1.02 ng/ml, as shown in Table 1) may exist in non-glucuronide-conjugate form in urine. Songsermsakul et al. (2013) reported that ZEN was biotransformed in horses, mainly to β-ZOL and α-ZOL in a mean ratio of 1.4:1, and the degree of glucuronidation of ZEN and its metabolites excreted in urine in glucuronide-conjugate form was approximately 100%. The reason for the difference in conjugation status of ZEN between the present and previous studies remains to be elucidated, but breed differences (horse breeds) and differences in enzymatic digestion treatment method (differences in supplemented volume of β-glucuronidase, pre-incubation time, etc.) may account for the apparent discrepancy. Thus, further studies are warranted to identify the biotransformation of ZEN in horses in more detail, as the oestrogenic potency of the Phase 1 metabolites α- and β-ZOL varies significantly. It is estimated that α-ZOL is about 60 times more potent than the parent ZEN, whereas the β-ZOL potency factor is 0.2 (compared to the parent ZEN). The ratio of α- and β- ZOL is considered to be an indication of the sensitivity of individual animal species to ZEN exposure. The presented results show that horses apparently produce predominantly β-ZOL, and hence, horses can be classified as less sensitive to the adverse effects of ZEN than, for example, pigs (the rate α-ZOL/β-ZOL; 2–3:1) as reported previously (Malekinejad, Maas-Bakker, & Fink-Gremmels, 2005).

Of clinical importance is the observation that the urinary levels of ZEN and its metabolites were not directly correlated to the conception rate of female horses. However, this study included only a few mares (n = 7) that did not conceive. Moreover, it needs to be considered that urinary samples reflect only very recent exposure to mycotoxins in the days before the analysis, as the half-life of ZEA is rather short (Dänicke & Winkler, 2015) although precise data for horses are lacking. Hence, we cannot entirely exclude the possibility that long-term exposure to the mycoestrogen ZEN causes early reduction of fertility in horses.

Another objective of this study was to clarify the contamination status of STC on the farms. Our results indicated that the number of STC-positive urine samples was rather low, and the concentration of STC was also low. STC urine concentrations ranged 1.3-18.0 (pg/mg Crea). The lower prevalence of STC reflects a good hygienic status of the feed, as STC is generally considered as a mycotoxin resulting from post-harvest (storage) contamination. In Japan, there is no regulation for maximal tolerable limits of STC in feed, and in the absence of toxicological data on the STC tolerance of horses, the clinical relevance of the current findings remains unknown. Vesonder and Horn (1985) previously reported acute clinical signs, including bloody diarrhoea and death, in dairy cattle given STC-contaminated (8 mg/kg) feed infected with *A. versicolor*.

In conclusion, the results from urinalyses of thoroughbred mares indicated that previously established methods, developed for cattle, could also be successfully applied to horses. This is the first study to show that STC can exist in the feed of horses, and confirms the relative abundance of ZEN in equine diets. Further studies should focus on the relationship between mycotoxin concentrations in feed materials and resultant urinary concentrations to predict the rate of absorption. Moreover, the results indicate that animal diets in many cases contain more than one mycotoxin, and that these mycotoxins may result from both pre- and post-harvest contamination of feed materials. Therefore, the potential adverse effects of such complex mixtures of mycotoxins deserve further investigation.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

M. Takagi and J. Fink-Gremmels conceived and designed the experiments; M. Takagi, S. Uno, E. Kokushi and F. Sato performed the experiments; M. Takagi, S. Uno and E. Kokushi analysed the data; F. Sato and MMP. Wijayagunawardane contributed reagent/material/analysis tools; all authors contributed to the drafted manuscript.

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