Field Application of Urinary Zearalenone and Sterigmatocystin Measurements for Monitoring Natural Feed Contamination in Cattle Herds: Effects of Mycotoxin Adsorbent and Affect on Reproductive Functions

（尿中濃度を指標とした飼養環境下の牛群におけるゼアラレノンとステリグマトシスチン浸潤動態：マイコトキシン吸着剤の投与効果と繁殖機能への影響）

The United Graduate School of Veterinary Science

Yamaguchi University

Yasuo Fushimi

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By

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We hereby recommend that the thesis prepared under supervision by Yasuo Fushimi, entitled “Field Application of Urinary Zearalenone and Sterigmatocystin Measurements for Monitoring Natural Feed Contamination in Cattle Herds: Effects of Mycotoxin Adsorbent and Affect on Reproductive Functions” should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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TABLE OF CONTENTS

ABSTRACT

The first series of experiments were conducted to investigate (1) protective effects of a commercially available mycotoxin adsorbent (MA) and (2) endocrine effects of *in vivo* exposure to zearalenone (ZEA) in cattle. The sample included a Japanese Black female cattle herd (MYT herd) that displayed persistently high urinary ZEA concentrations. A second herd (NM herd) was used as a control. Three groups from each herd were assessed: MX (n = 6; MA mixed with concentrate), TD (n = 6; MA applied as topdressing with the concentrate), and a positive control (n = 6; no MA application). Urine and blood samples were collected at the start of MA supplementation (Day 0), on the final day of supplementation (Day 16), and on the final day of the sampling period (Day 58 for MYT herd and Day 50 for NM herd). Urinary ZEA concentrations (pg/mg of creatinine) were measured by ELISA and tandem mass spectrometry (LC/MS/MS). Haematological and serum biochemical analyses were performed to monitor hepatic, renal, nutritional, and mineral intake statuses. Ovulation status was assessed by progesterone (P4) and antral follicle population by anti-Müllerian hormone (AMH) levels. The urinary concentrations of ZEA and its metabolites in the MX and TD groups were significantly lower ($P < 0.05$) at Day 16 compared with the control group when measured using LC/MS/MS. The valid ratio of AMH-positive (>0.08 ng/mL) cattle was significantly higher in NM herd than MYT herd without affecting the P4-positive (>3 ng/mL) ratio, suggesting different populations of antral follicles. Significant differences were also observed between the MX and control in aspartate aminotransferase and γ-glutamyltransferase at Day 58, suggesting the preventive

effects of MA supplementation. Our field trial indicates that MA supplementation of a ZEA-contaminated diet has beneficial effects in reducing ZEA absorption from the intestine of cattle, maintaining endocrine homeostasis and reversing hepatic effects.

In the second experimental series, we investigated the effects of *in vivo* exposure to low zearalenone levels on the anti-Müllerian hormone endocrine levels and the reproductive performance of cattle. Urine and blood samples and reproductive records were collected from two Japanese Black breeding female cattle herds with dietary zearalenone contamination below the threshold levels (< 1 ppm) at 30 d after calving. Urinary zearalenone, α-zearalenol, and β-zearalenol concentrations were measured by chromatography–tandem mass spectrometry, and serum anti-Müllerian hormone concentrations were determined along with serum biochemical parameters. Urinary concentrations of α- zearalenol were significantly higher ($p < 0.05$) in cattle in Herd 1 than in cattle in Herd 2, reflecting the different amounts of zearalenone in the diet of the two herds. Although the number of 5-mm and 10-mm follicles of the herds and their fertility after artificial insemination were similar, the serum anti-Müllerian hormone concentrations in Herds 1 and 2 were 438.9 ± 48.6 pg/mL and 618.9 ± 80.0 pg/mL, respectively, with a trend toward a significant difference ($p = 0.053$), which may indicate differences in the antral follicle populations between herds. Thus, zearalenone intake from dietary feed, even when below the threshold zearalenone contamination level permitted in Japan, may affect the ovarian antral follicle populations, but not the fertility, of postpartum cows.

The third series of experiment aimed (1) to determine the levels of the fungal toxin sterigmatocystin (STC) in the feed and urine of cattle and (2) to evaluate the effects of supplementing the feed with a mycotoxin adsorbent (MA) on STC concentrations in urine. Two herds of female Japanese Black cattle were used in this study. The cattle in each herd were fed a standard ration containing rice straw from different sources and a standard concentrate; two groups of cattle from each herd (n = 6 per group) received the commercial MA, mixed with the concentrate or given as top-dressing, whereas a third group received no supplement and served as control. Urine and feed samples were collected at various time points throughout the experiment. STC concentrations were measured using liquid chromatography-tandem mass spectrometry. STC concentrations in straw were higher in herd 1 (range: 0.15–0.24 mg/kg DM) than in herd 2 (range: <0.01–0.06 mg/kg DM). In Herd 1, STC concentrations in urine significantly declined 2 wks after replacing the contaminated feed, whereas MA supplementation had no effect. In conclusion, mycotoxins in urine samples are useful biological markers for monitoring the systemic exposure of cattle to multiple mycotoxins, as well as evaluating the effectiveness of interventions.

In conclusion, the results of these field trials confirm that a system for monitoring urinary ZEN and STC concentrations is a useful tool for predicting the exposure of animals at the farm level to ZEN and STC. By applying the urinary monitoring system, it allows for the objective evaluation of MA supplementation to feeds at the farm level. Moreover, dietary contamination with mycotoxins may affect the population of antral follicle in the ovary.

GENERAL INTRODUCTION

Mycotoxins are secondary metabolites produced by specific filamentous fungi that are common contaminants of agricultural commodities, and are known to induce a toxic response (mycotoxicosis) when ingested (Binder et al; 2007; Boudra and Morgavi, 2008). It is well known that naturally contaminated feed on the field could contain many different mycotoxins (more than 300 different mycotoxins have been reported thus far): however, the major class of mycotoxins affecting feedstuffs includes aflatoxins, deoxynivalenol (DON), and zearalenone. These contaminants are present at the highest levels in corn, an ingredient of concentrate feeds (Binder et al, 2007). In the case of farm animals, consumption of a mycotoxin-contaminated feed may induce acute and chronic effects, such as reduced feed intake, feed refusal, poor feed conversion, diminished body weight, increased disease incidence, and reduced reproductive efficacy, resulting in economic losses of each herd (Fink-Gremmels and Malekinejad, 2007; Binder et al, 2007).

Based on the worldwide surveillance to evaluate the incidence of mycotoxins in feeds sampled from animal farms or feed production sites, more than half of the samples in Europe were reported to be contaminated at levels above the quantification limit of current methods, and one-third of the samples from Asia-Pacific regions were positive (Binder et al, 2007). Therefore, monitoring for the contamination status of mycotoxins in dietary feeds of farm animals, especially at the farm levels, is an essential measure to prevent feed contamination. Exposure to mycotoxins is usually assessed by analyzing feed materials of total mixed rations. An alternative approach is the measurement of toxin concentrations in biological samples

(such as urine) that reflect the individual exposure levels (Prelusky et al., 1989; Solfrizzo et al., 2011). Previously, we established a urine monitoring system for the mycotoxin zearalenone (ZEN) and its metabolites in cattle (Takagi et al., 2011; Hasunuma et al., 2012)

Zearalenone (ZEN) is a resorcylic acid lactone produced as a secondary metabolite by various *Fusarium* species, including *F. graminearum*, *F. culmorum,* and *F. cerealis,* which are all common soil fungi infecting plants in the early stage of growth and during flowering (Jimenez and Mateo, 1997; Malekinejad, 2004). ZEN has also been found in legumes, oats rice, and sorghum, as well as in various grass species. Since the lactone ring of ZEN resembles many structural features of the aromatic ring of estradiol, and apparently fits into binding pocket of mammalian estrogen receptor, ZEN and its metabolites exhibit distinct estrogenic and anabolic properties that affect the reproductive system of several animal species (Kleinova et al., 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell'Aquila, 2008).

Sterigmatocystin (STC) is a fungal secondary metabolite produced by fungi of the genera *Aspergillus* and *Penicillium*. STC is the end product of a biosynthetic pathway in some fungal species such as *A. versicolor* and *A. nidulans*, but is also a well-known precursor of aflatoxin B1 synthesis in various other fungal species (Hsieh et al., 1973; Wilkinson et al., 2004; Versilovskis et al., 2010). STC has been shown to be genotoxic and potentially carcinogenic in studies with experimental animals (EFSA, 2013), and exerts teratogenic effects at higher exposure levels (Kusunoki et al., 2011). The European Food Safety Authority (EFSA) recently concluded that despite its potential carcinogenic effects, STC is of minor concern to human health in Europe due to its limited prevalence in European food

commodities and subsequently a high margin of exposure (MoE) index. However, at the same time, the EFSA concluded that there were not sufficient data to draw conclusions about animal exposure and potential adverse health effects in animals, including cattle (EFSA, 2013). STC is frequently reported as a contaminant in feeds (e.g., grains, maize, and rice straw), but data on its adverse health effects in cattle is scarce and hence maximum exposure limits have not been established.

Several methods have been proposed for the mitigation of mycotoxin contamination of feeds during the preharvest, harvest, and postharvest periods (Kabak et al., 2006; Schatzmayr et al., 2006; Igawa et al., 2007; Zinedine et al., 2007; Boudra and Morgavi, 2008; Murata et al., 2008). Given the various limitations of these methods, it has been suggested that the use of mycotoxin adsorbents (MAs), MA-related mitigating agents, or both, as feed additives is one of the most promising and widely used approaches to reduce the risk of mycotoxicosis in farm animals (Ramos et al., 1996; Huwig et al., 2001; Sabater-Vilar et al., 2007). To date, classes of substances suggested to be MAs include inorganic mineral clays and zeolites, as well as organic products such as yeast cell wall constituents, activated charcoal, humic acid polymers, and micronized plant fibers (Ramos et al., 1996; Jouany, 2007; Santos et al., 2011). Several *in vitro* and *in vivo* studies of the efficacy of MA have shown beneficial effects of MAs (Avantaggiato et al., 2005, 2007; Korosteleva et al., 2007; Sabater-Vilar et al., 2007). However, to our knowledge, no study in farm animals has confirmed the absorptive capacity *in vivo* by measuring ZEN levels in biological fluids (such as milk, urine, bile, and serum) under field conditions to demonstrate a reduced rate of absorption after MA supplementations.

Anti-Müllerian hormone (AMH) is a glycoprotein exclusively produced by the granulosa cells of growing ovarian follicles in the adult female (Vigier et al. 1984; Monniaux et al. 2013). AMH is a key factor that inhibits the recruitment of primordial follicles into the pool of growing follicles and decreases the responsiveness of growing follicles to follicle-stimulating hormone (Durlinger et al. 2002; Visser and Themmen 2014). Currently, AMH is considered to be the best endocrine marker of the population of small antral gonadotropin-responsive follicles in cows (Rico et al. 2009; Ireland et al. 2011; Monniaux et al. 2014). In female cattle, ovarian AMH expression is restricted to the granulosa cells of healthy small antral follicles and is strongly decreased when the follicles enter atresia (Rico et al. 2009, 2011). A previously study demonstrated in an *in vitro* culture system that ZEA and its metabolites can induce the apoptosis of granulosa cells in a dose-dependent manner via a caspase-3- and caspase-9-dependent mitochondrial pathway (Zhu et al. 2012), resulting in follicular atresia. Altogether, these observations suggest that the difference in AMH endocrine levels between herds reflects a difference in the atresia rate of the populations of small antral ovarian follicles, which are affected by different ZEA contamination levels. In any case, ZEA, at least at low dietary contamination levels, would not modulate terminal follicular development and ovulation, and would not affect the fertility of cows with low contamination levels.

Therefore, the Chapter 1 was conducted (1) to evaluate the supplementary effects of MA on cattle through urinary concentrations of ZEA and its metabolites, and (2) to obtain preliminary knowledge regarding the effects of *in vivo* exposure to ZEA and its metabolites on ovarian function assessed by measurements of serum AMH and progesterone as well as on blood biochemical parameters, taking into account method of MA supplementation (mix or topdressing) with dietary feed. Additionally, in the Chapter 2, we investigate the effects of *in*

vivo exposure to low ZEA contamination levels (below the threshold levels mandated in Japan) on the serum AMH concentration of breeding cattle herds, with regard to their reproductive performance. In the Chapter 3, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) of urine samples collected from these cattle herds to investigate the potential co-exposure to STC. We also used this approach to assess the efficacy of a commercial mycotoxin adsorbent.

Chapter 1

Application of mycotoxin adsorbent to cattle feed naturally contaminated with zearalenone: Urinary zearalenone excretion and association with anti-Müllerian hormone

ABSTRACT

This study investigated (1) protective effects of a commercially available mycotoxin adsorbent (MA) and (2) endocrine effects of *in vivo* exposure to zearalenone (ZEA) in cattle. The sample included a Japanese Black female cattle herd (MYT herd) that displayed persistently high urinary ZEA concentrations. A second herd (NM herd) was used as a control. Three groups from each herd were assessed: MX (n = 6; MA mixed with concentrate), TD (n = 6; MA applied as topdressing with the concentrate), and a positive control (n = 6; no MA application). Urine and blood samples were collected at the start of MA supplementation (Day 0), on the final day of supplementation (Day 16), and on the final day of the sampling period (Day 58 for MYT herd and Day 50 for NM herd). Urinary ZEA concentrations (pg/mg of creatinine) were measured by ELISA and tandem mass spectrometry (LC/MS/MS). Haematological and serum biochemical analyses were performed to monitor hepatic, renal, nutritional, and mineral intake statuses. Ovulation status was assessed by progesterone (P4) and antral follicle population by anti-Müllerian hormone (AMH) levels. The urinary concentrations of ZEA and its metabolites in the MX and TD groups were significantly lower ($P < 0.05$) at Day 16 compared with the control group when measured using LC/MS/MS. The valid ratio of AMH-positive (>0.08 ng/mL) cattle was significantly higher in NM herd than MYT herd without affecting the P4-positive (>3 ng/mL) ratio, suggesting different populations of antral follicles. Significant differences were also observed between the MX and control in aspartate aminotransferase and γ-glutamyltransferase at Day 58, suggesting the preventive effects of MA supplementation. Our field trial indicates that MA supplementation

of a ZEA-contaminated diet has beneficial effects in reducing ZEA absorption from the intestine of cattle, maintaining endocrine homeostasis and reversing hepatic effects.

INTRODUCTION

Zearalenone (ZEA) is a nonsteroidal oestrogenic mycotoxin produced by *Fusarium* species in plants including pasture grasses. ZEA and its metabolites exhibit distinct oestrogenic and anabolic properties that affect the reproductive system of several animal species (Fink-Gremmels and Malekinejad, 2007; Kleinova *et al.*, 2002). These substances are generally accepted as endocrine disrupters (Fink-Gremmels and Malekinejad, 2007). Several *in vivo* trials and *in vitro* assays have been conducted thus far to determine the effects of ZEA and its metabolites on reproductive function of cattle (Takagi *et al.*, 2008; Weaver *et al.*, 1986a, 1986b), and the results largely support the above hypothesis, although some contradictions still exist.

Several approaches for protecting animals from the toxic effects of natural mycotoxin contamination from both pre- and post-harvested products have been reported (Awad et al., 2010). Among these approaches, supplementation of feed with mycotoxin adsorbents (MA) has been recognized as an effective and practical approach at the farm level (Kutz *et al.*, 2009; Sabater-Vilar *et al.*, 2007). MA generally contain a mineral clay carrier, yeast cell wall preparations, and, in some cases, enzymes or living microorganisms (probiotics) that may adsorb and detoxify mycotoxins. We recently reported the effects of MA in cattle herds, and indicated the significant effects of this MA for reduction of urinary ZEA concentrations concomitantly with reduction of somatic cell counts in a dairy cattle herd (Takagi *et al.*, 2011).

The objectives of this field study were (1) to evaluate the supplementary effects of MA on cattle through urinary concentrations of ZEA and its metabolites, and (2) to obtain preliminary knowledge regarding the effects of *in vivo* exposure to ZEA and its metabolites on ovarian function assessed by measurements of serum anti-Müllerian hormone (AMH) and progesterone as well as on blood biochemical parameters, taking into account method of MA supplementation (mix or topdressing) with dietary feed.

MATERIALS AND METHODS

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (Faculty of Agriculture, Kagoshima University).

Chemicals and solvents

ZEA was purchased from MP Biomedicals (Heidelberg, Germany). The metabolites α-zearalenol (ZOL) and β-ZOL were purchased from Sigma (St. Louis, MO). Stock solutions of ZEA, α-ZOL, and β-ZOL, each at a concentration of 1 μg/mL in methanol, were stored under light protection 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/arylsulphatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan) and Tris was purchased from Nakalai Tesque Inc. (Kyoto, Japan).

Japanese Black cattle herds and sample collection

During our series of field studies, we accidentally detected one cattle herd that showed significantly higher urinary ZEA concentrations than other herds (Takagi *et al.*, 2011). Afterwards, our investigations revealed that contaminated rice straw used in this herd was the source of the high urinary ZEA concentrations detected (Hasunuma *et al.*, 2012). During the process of searching for the originally unknown cause of the high urinary ZEA concentrations of this herd, we applied a supplementary MA within the dietary feed of the herd, not only to

protect the cattle from ZEA contamination but also to clarify the effects of MA supplementation based on urinary ZEA concentrations, because we considered the herd a useful model of ZEA contamination under farm conditions.

Two herds (MYT herd and NM herd) of Japanese Black female cattle kept for fattening in Kagoshima Prefecture, Japan, were included in this study, as partly reported previously (Hasunuma *et al.*, 2012). MYT herd, with a herd size of 700 animals, showed persistently significantly higher mean urinary ZEA concentrations than 7 other herds (3 fattening and 4 breeding herds) studied previously (Takagi et al., 2011). NM herd, with a herd size of 120, was one of the above 3 fattening herds with significantly lower urinary ZEA concentrations than MYT herd. NM herd was fed with the same lot of concentrate purchased from the same company as that used for MYT herd, but received different straw and water. Most of the straw fed to the cattle in MYT herd was commercially available straw purchased from a local fodder supplier, and the straw used for NM herd was purchased directly from local rice farmers. In both herds, the straw and concentrates were stored in feed sheds and silos, respectively, at ambient temperature.

Because the significantly high urinary ZEA concentrations observed in the animals of MYT herd were determined several months before the present study, preliminary monitoring was conducted in both herds (n = 5 from each herd) 1 week before (Day -7) the start of MA supplementation to reconfirm urinary ZEA concentrations; in addition, preliminary monitoring included assessment of the ZEA concentrations of feed and water samples from both herds. All of the animals from both herds were housed indoors with 2 cows per pen (MYT herd, 4 m $\times$ 3 m; NM herd, 3 m $\times$ 3 m), and were fed straw and concentrates separately.

The composition of the feed given to each herd has been described previously (Hasunuma *et al.*, 2012).

Three groups of the same age (23 months) and similar body weight (range 550 – 600 kg) were randomly selected from both herds and treated as follows: MX (n = 6; MA mixed with concentrate), TD (n = 6; MA topdressed on concentrate), and control (n = 6; no MA supplementation). The supplemented MA (Mycofix®Plus3.E, Biomin) was a commercially available product comprising minerals and biological constituents, including enzymes, yeast cell wall, clay, and plant extracts (Marroquin-Cardona et al., 2009). The maximum daily dose of MA recommended by the supplier (50 g) was given as a twice-daily divided dose for a 16-day period.

At 2 h after the morning feed, spontaneous urine samples were collected from the animals by massaging the pudendum at the start of supplementation (Day 0), on the final day of supplementation (Day 16), and on the final day of sampling (Day 58 for MYT herd and Day 50 for NM herd). Blood samples from the jugular vein were also collected from all animals concomitantly with urine sampling; 2 mL samples were collected in EDTA tubes for haematological analysis and 10 mL were collected in silicone-coated tubes for serum biochemistry analysis. In addition, samples of approximately 1 kg each of rice straw and concentrate feed and 50 mL of water were obtained from both herds to measure ZEA concentrations. The scheme of the MA supplementation and all sampling procedures conducted in the present study is summarized in Fig. 1. All samples were immediately placed into a cool box containing ice, for protection from light, and transported to the laboratory. After centrifugation at $500 \times g$ for 10 min at room temperature, urine and blood samples were

frozen at -30°C until analysis of ZEA and creatinine (Crea) concentrations in urine samples and biochemical parameters in serum samples. The feed samples were frozen at -30°C until analysis of ZEA concentrations. The urinary ZEA concentrations were primarily measured by ELISA, and for confirmation, the concentrations of urinary ZEA, α-ZOL, and β-ZOL were re-measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The ZEA concentrations of feed samples were measured using HPLC with fluorescence detection according to the methods of Emoto et al. (2008).

Analytical methods of ZEA and Crea measurement

ZEA concentrations in urine were determined using a commercially available kit (RIDASCREEN Zearalenon; R-Biopharm AG, Garmstadt, Germany) according to the manufacturer's instructions with minor modifications, and the urinary ZEA concentrations were expressed as a ratio to urinary Crea (urinary ZEA/urinary Crea (pg/mg Crea)), as described previously (Takagi et al., 2011). The cross reactivity rates using this particular ELISA kit for α- and β-ZOL were 41.6% and 13.8%, respectively, based on the manufacturer's instructions.

All urine samples evaluated by ELISA were re-analysed using LC/MS/MS, which confirmed the measured ZEA and ZEA metabolite urine concentrations. Analyses with LC/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), and the urinary ZEA concentrations were expressed as urinary ZEA/Crea ratio as described above. The detection limits for ZEA,

α-ZOL, and β-ZOL were 0.04 ng/mL, 0.05 ng/mL, 0.05 ng/mL, respectively, and the mean recovery rates for ZEA, α-ZOL, and β-ZOL were 116%, 121%, and 56%, respectively. In the present study, the final urinary ZEA and metabolite concentrations were not corrected using the respective recovery rates.

Endocrinological parameters analysis

To compare the size of the ovarian pool of growing follicles between MYT herd and NM herd, serum concentrations of AMH of the selected serum samples, MX and control groups from both MYT and NM herds, were measured using a commercially available AMH Gen II ELISA kit (Beckman Coulter, Inc., CA) according to the manufacturer's instructions. The sensitivity of this assay was 0.08 ng/mL, and the intra-assay coefficients of variation were 1.4% and 4.1% for quality control plasma samples containing 2.8 and 7.9 ng/mL of AMH, respectively. In the present study, the samples whose AMH concentrations were more than 0.08 ng/mL were considered valid, and not only the ratio of valid samples but also the mean AMH concentrations of the valid samples were compared between the 2 herds. Additionally, with the same samples, the serum progesterone (P4) concentrations of each animal were measured using double-antibody EIA in 96-well ELISA plates according to a previous report (Miyamoto et al., 1992). This was performed to monitor the occurrence of luteinisation (ovulation) in each animal from both herds during the period between the first and third sampling points. The sensitivity of this P4 assay was 0.05 ng/mL, and the average intra- and inter-assay coefficients of variation were 6.5% and 9.7%, respectively. For the final evaluations, only the data from control animals of both herds were calculated to avoid the

effect of MA supplementation in the present study.

Haematological and serum biochemical analyses

For the haematological examinations, red blood cell count (RBC), white blood cell count (WBC), and haemoglobin (Hb) concentration were determined using an automatic blood cell counter (F-820; Sysmex, Kobe, Japan), and packed cell volume (PCV) was determined using a microhaematocrit centrifuge and a haematocrit reader. Serum biochemical analysis was performed to monitor the hepatic, renal, and nutritional statuses as well as the mineral intake of the animals. Biochemical analysis was performed to determine the following parameters: glucose (Glu), free fatty acid (FFA), and total cholesterol (T-Cho) for evaluation of energy metabolism; total protein (TP), serum albumin (Alb), and blood urea nitrogen (BUN) for protein metabolism; serum aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) for liver function; and calcium (Ca), magnesium (Mg), and iron (Fe) for mineral metabolism (measured on a Labospect 7080 autoanalyser; Hitachi, Tokyo, Japan). Additionally, the plasma insulin-like growth factor-1 (IGF-1) concentration was determined to evaluate the metabolic function of the liver by using enzyme immunoassay with the biotin-streptavidin amplification technique (Kawashima et al., 2007).

Statistical analysis

The results for ZEA and its metabolite concentrations as well as endocrinological, haematological and serum biochemical parameters were expressed as mean ± SEM. The concentrations of urinary ZEA, α-ZOL, β-ZOL, and total ZEA (ΣZEA; ZEA + α-ZOL +

β-ZOL) concentrations collected at 3 sampling points from each herd were analysed among the 3 groups (MX, TD, and control) by a 1-way ANOVA followed by a post hoc test using the StatView program (Abac Concepts, Inc., Berkley, CA) to evaluate the effect of MA supplementation. Similarly, the results of serum AMH concentrations of the valid animals and serum biochemical analysis were compared with the same procedures to monitor the effects of ZEA exposure on the antral follicle population of the ovaries, and to determine the effects of MA supplementation in herds with and without ZEA contamination, respectively. The valid ratio of the animals whose AMH concentrations were more than 0.08 ng/mL was analysed by chi-square test with Fisher's exact probability. *P* values less than 0.05 were considered to indicate a statistically significant difference, while P values less than 0.1 were considered to indicate a significant tendency.

RESULTS

Urinary and dietary feed ZEA concentrations during the MA supplementation period measured by ELISA

Urinary ZEA concentrations were measured at 1 week before (Day -7) the start of MA supplementation in both herds (n = 5). Concomitantly, ZEA was measured in water samples from both herds. This revealed that the urinary ZEA concentrations of cattle in MYT herd remained high; the mean urinary ZEA concentration (n = 5) was 8,887 ± 1,212 pg/mg Crea (range, 5,268 – 11,697 pg/mg Crea). On the other hand, the mean ZEA concentration in NM herd (n = 5) was 323 ± 81 pg/mg Crea (range, 126 – 588 pg/mg Crea). Furthermore, the ZEA concentrations of water samples collected from MYT herd (48 pg/mL) and NM herd (81 pg/mL) were equivalent to those of commercially available distilled water (62 pg/mL), which was used as a non-contaminated control. Thus, more severe ZEA contamination of the dietary feed used in MYT herd was confirmed at 1 week before starting the MA supplementation study.

The results for urinary ZEA concentrations measured by ELISA and dietary feed sample ZEA concentrations measured by HPLC are shown in Table 1. In MYT herd, although urinary ZEA concentration was significantly lower in the TD group (906 pg/mg Crea) than in both the MX (1,958 pg/mg Crea) and control (2,688 pg/mg Crea) groups on Day 0, no significant differences were observed on either Day 16 (after MA supplementation) or Day 58 (42 days after stopping MA supplementation on Day 16). Regarding the ZEA concentrations of dietary feeds, the straw samples collected on Day 16 and Day 58 showed ZEA

concentrations of more than 7000 ng/g, which were reflected by the urinary ZEA concentrations on those days. The ZEA concentrations measured on Day 0 were 150 times lower than those in the 2 samples collected on Days 16 and 58. On the other hand, in NM herd, no significant differences were observed among the 3 groups of urinary samples collected on Day 0, Day 16 (after MA supplementation), and Day 50 (34 days after stopping MA supplementation on Day 16), except between the TD (486 pg/mg Crea) and control (334 pg/mg Crea) groups on Day 16 ($P < 0.05$).

Urinary ZEA concentrations during the experimental periods of MA supplementation measured by LC/MS/MS

The results for the urinary concentrations of ZEA, α-ZOL, and β-ZOL measured by LC/MS/MS in MYT herd are shown in Fig. 2. Tendencies, which reflected the ZEA concentrations of the dietary straw, similar to those seen with the results of ELISA assay were observed; namely, the urinary ZEA concentrations on Day 0 (MX, 720 ± 414; TD, 415 ± 42; control, 457 ± 59 pg/mg Crea), which were not significantly different among the 3 groups, were low compared with the samples collected on Day 16 (MX, 2,444 ± 394; TD, 4,125 ± 452; control, 8,166 ± 1,979 pg/mg Crea) and Day 58 (MX, 2,284 ± 457; TD, 2,494 ± 618; control, 4,011 ± 434 pg/mg Crea). Significant differences ($P < 0.05$) were observed among the MX, TD, and control groups for ZEA and α-ZOL and between the MX and control groups for β-ZOL and total ZEA on Day 16. Moreover, a significant difference ($P < 0.05$) was observed between the MX (3,702 ± 747 pg/mg Crea) and control (7,205 ± 812 pg/mg Crea) groups on Day 58.

The results for the urinary concentrations of ZEA, α-ZOL, and β-ZOL measured by LC/MS/MS in NM herd are shown in Fig. 3. No significant differences were observed on Day 0 among the 3 groups. On Day 16, the ZEA concentrations of both the MX (68 pg/mg Crea) and TD (65 pg/mg Crea) groups were significantly lower ($P < 0.01$) than those of the control group (349 pg/mg Crea), but no significant differences were observed in urinary concentrations of α-ZOL, β-ZOL, or total ZEA. On Day 50 (34 days after stopping MA supplementation), no significant differences were observed in urinary concentrations of ZEA, α-ZOL, β-ZOL, or total ZEA among the 3 groups. The ZEA concentrations of the straw collected at the 3 sampling points were stable in NM herd.

Serum AMH and P4 concentrations during the experimental period

To monitor both the number of growing antral follicles and the occurrence of follicular ovulation within the ovaries of each herd, both the serum AMH and P4 concentrations of each animal, which were assessed at each sampling point, were measured. The percentages of valid cattle whose AMH concentration was more than the detection limit (0.08 ng/mL) and the mean AMH concentrations of the valid samples are shown in Table 2. The total number of samples measured was 11 in MYT herd (MX, 6 and control, 5) and 11 in NM herd (MX, 6 and control, 5) because serum samples were not available for 1 animal in the control group of each herd. The number of valid cattle in control in NM herd at Day 50 was significantly higher ($P<0.05$) than that of both MX and control in MYT herd at Day 58. Additionally, differences (but not statistically significant) were noticed between control in MYT herd and control in NM herd at Day 0 ($P = 0.09$), between MX in MYT herd and control in NM herd at

Day 16 (P = 0.06), and between MX in MYT herd and MX in NM herd (P = 0.08) and between control in MYT herd and MX in NM herd (P = 0.07) at Day 58 or Day 50. However, the mean AMH concentration of the valid samples of MYT herd and NM herd did not differ significantly between the herds at each sampling point. Regarding the criteria for P4 evaluation used in the present study, the animals whose serum P4 concentrations were more than 3.0 ng/mL in at least one sample of the 3 consecutive samples were considered to be ovulating. In the present study, the results for P4 concentrations in 5 out of 6 animals from both herds were available, and all of these 5 animals from each herd exhibited P4 concentrations of more than 3.0 ng/mL (maximum P4 concentration, 6.3 ng/mL in MYT herd and 5.0 ng/mL in NM herd) at one or more sampling points.

Haematological and serum biochemical analyses during the experimental periods of MA supplementation

The results of the haematological and serum biochemical analyses in MYT herd and NM herd are shown in Table 3 and Table 4, respectively. In both herds, no significant differences in any of the parameters measured on Day 0 were confirmed among the 3 groups. In MYT herd, no significant differences were observed among the 3 groups for the haematological, protein metabolism, and energy metabolism parameters or IGF-1 at each of the sampling points. Significant differences were observed between the MX and control groups in AST (74.2 and 161.7 IU/L, respectively) and GGT (36.3 and 70.7 IU/L, respectively) on Day 58, and in Mg (2.1 and 1.8 mg/dL, respectively) on Day 16. In NM herd, no significant differences were observed among the 3 groups for liver function parameters or

IGF-1 at each of the sampling points. Significant differences were observed in Alb (between MX, 3.8 g/dL, and TD, 3.5 g/dL, on Day 16), FFA (between TD, 198.8 μEq/L, and control, 352.6 μEq/L, on Day 16), RBC (between MX, 919×10^4, and control, 813×10^4, on Day 16), Glu (between MX, 65.4 mg/dL, and control, 73.3 mg/dL, on Day 50), and Fe (between MX, 149.3 mg/dL, and TD, 206.8 mg/dL, on Day 50).

DISCUSSION

We have previously reported that monitoring urinary ZEA concentrations could be useful not only for the detection of exposure of cattle to ZEA contamination, but also for the *in vivo* evaluation of MA efficiency (Hasunuma *et al.*, 2012; Takagi *et al.*, 2011). Moreover, regarding MA supplementation, we suggested that the method used to supply MA with feeds (mixing or topdressing) might have a significant effect on the extent of absorption of mycotoxin from the gastrointestinal tract, and may be a possible limitation of MA efficiency (Hasunuma *et al.*, 2011). Thus, one of the primary aims of the present study, conducted on a herd exposed to ZEA with naturally contaminated rice straw, was the evaluation of MA efficacy by using our established urinary ZEA monitoring methods. We also aimed to clarify the effects of ZEA contamination on serum biochemical parameters of affected cattle compared with non-affected control cattle. Regarding these points, our results indicate the following conditions of MA supplementation: Although MA supplementation might be effective in reducing the absorption of ZEA and its metabolites from the intestine of cattle, this effect might be dependent upon the method of application (mixing or topdressing). Furthermore, the effect of MA supplementation is associated with the degree of mycotoxin contamination of feed materials.

It has been suggested that the use of MA is one of the most promising and widely used approaches to reduce the risk of mycotoxicosis in farm animals (Huwig *et al.*, 2001; Ramos *et al.*, 1996; Sabater-Vilar *et al.*, 2007). To date, the classes of substances suggested to be MA include inorganic mineral clays and zeolites, as well as organic products such as yeast cell wall constituents, activated charcoal, humic acid polymers, and micronized plant fibres

(Jouany, 2007; Ramos *et al.*, 1996; Santos et al., 2011). Recently, we also reported a field study that confirmed a significant reduction in urinary ZEA concentration after a period of MA supplementation (2 weeks) in a dairy herd, which was followed by a subsequent increase in ZEA levels after cessation of MA supplementation (Takagi *et al.*, 2011). On the other hand, recent reports have suggested that MA types, as well as application methods, might affect absorption of mycotoxins in the digestive tract (Kutz *et al.*, 2009). Although supplementary MA is usually applied to animal diets as homogeneous mixtures in total mixed rations (TMR) in the dairy industry, another possible method for MA supplementation is as a topdressing on the feed of individual cattle. Hasunuma *et al.* (2011) investigated the effect of MA supplementation as a topdressing on feed by using measurements of urinary concentrations of ZEA and its metabolites at the farm level, and indicated that there was no reduction in the concentrations of urinary ZEA or α-ZOL and β-ZOL after supplementation with MA in 4 cattle herds without ZEA contamination. Regarding the effects of MA supplementation in the present study, even though significant effects of MA were obtained, it was impossible to reduce the urinary ZEA concentration levels to equal those of cattle fed with ZEA-uncontaminated rice straw. Although the supplementation method, whether mixed uniformly in a TMR or applied as a topdressing on the feed, may affect gastrointestinal absorption of ZEA and its metabolites in cattle, MA may have limited ability to bind to highly concentrated mycotoxin. Another possibility is that MA may bind to mycotoxins; however, this may depend on the type/degree of mycotoxin contamination in the feeds. Obviously, further study is required to further clarify the effects of MA, such as investigation of the supplemental volume of MA or types of MA supplementation.

An important aim of the present study was to clarify the chronic effects of ZEA exposure by using the results of serum biochemical analysis to monitor general metabolic statuses and ovarian follicular status to investigate potential endocrine disruption. To do so, ZEA-contaminated cattle (MYT herd) were compared with those from a non-contaminated control herd (NM herd). Regarding the results of the serum biochemical analysis, all of the cattle examined were Japanese Black cattle kept for fattening, whose dietary conditions were specific for this purpose and included nutritionally controlled serum vitamin A levels. Thus, the background of this special feeding system for Japanese Black cattle should be taken into account. Although significant differences in some of the parameters such as Mg in MYT herd and Alb, FFA, Glu, Fe, and RBC in NM herd were observed in the present study, the values were almost within the normal physiological ranges of each parameter. These results suggested that a 2-week period of MA supplementation may itself cause moderate/slight changes in the metabolic functions of cattle with and without ZEA exposure. Interestingly, our results for both AST and GGT on Day 58 in MYT herd indicate possible beneficial effects of MA supplementation on recovery of liver function in ZEA-affected cattle with abnormal levels of each of these parameters. Although there are few reports available regarding the effects of ZEA and its metabolites on haematological and serum biochemical parameters of cattle under farm conditions, Seeling *et al.* (2006) previously reported the effects of *Fusarium* toxin intake (mainly deoxynivalenol [DON] and ZEA) on serum biochemical parameters of protein metabolism and liver damage in cows. They concluded that *Fusarium* toxin contamination of dietary feed (8.21 mg DON/kg DM [dry matter] and 0.09 mg ZEA/kg DM) was insufficient to induce toxicological changes in serum haematological variables such as

AST, GGT, TP, and Alb. It is generally accepted that the predominant feature of ZEA distribution in animal feed is its co-occurrence with other *Fusarium* toxins such as trichothecenes and fumonisins, and the levels of these mycotoxins can be noticeably high. Both ZEA and DON are originally derived from *Fusarium* species, and in many cases, both ZEA and DON coexist in contaminated feed and food (Avantaggiato *et al.*, 2007; Binder *et al.*, 2007). In the present study, we could not detect DON contamination, and we did not screen for toxins such as trichothecenes, fumonisins, and other mycotoxins, which may be produced by *Fusarium* species, in the ZEA-contaminated straw. Obviously, further investigations to clarify the status of coexistence of multiple mycotoxin contaminants within the ZEA-contaminated straw may bring new insights.

Interestingly, we preliminarily confirmed ZEA contamination in the rice straw on Day-7 before starting MA supplementation in MYT herd, but the ZEA concentrations both of straw and urinary samples collected on Day 0 were significantly lower than those of samples collected on Day 16 and Day 58, and almost the same as concentration levels in samples from NM herd. These results indicated not only that ZEA existed in 'spot like' distribution patterns within the same lot of contaminated rice straw but also the importance of the method of collection of dietary samples for monitoring ZEA concentration within stored feeds and, moreover, the usefulness of urinary ZEA monitoring systems under farm conditions.

Because the lactone ring of ZEA resembles many structural features of the aromatic ring of oestradiol, and apparently fits into the binding pocket of the mammalian oestrogen receptor, ZEA and its metabolites exhibit distinct oestrogenic and anabolic properties that affect the reproductive system of several animal species (Fink-Gremmels and Malekinejad,

2007; Kleinova *et al.*, 2002; Minervini and Dell' Aquila, 2008). It is generally accepted that AMH is a key factor in the ovary that inhibits the recruitment of primordial follicles into the pool of growing follicles and decreases the responsiveness of growing follicles to FSH (Rico *et al.*, 2009, 2011). Currently, AMH is considered the best endocrine marker of the population of small antral gonadotropin-responsive follicles in the cow (Rico *et al.*, 2009). Therefore, although the cattle examined in the present study were all for the purpose of fattening, AMH concentrations were compared between the ZEA-contaminated experimental female cattle from MYT herd and the low-contaminated female cattle from NM herd to monitor the effect of ZEA contamination on the population status of antral follicles in each cattle herd. Although chronic ZEA exposure (a level of approximately 8 ppm) in the dietary feed used in MYT herd did not significantly affect AMH concentrations without affecting the P4-positive (>3 ng/mL) ratio, compared with NM herd, the ratio of AMH-positive animals was significantly or tended to be higher in NM herd than MYT herd, suggesting that the population of antral follicles differed between MYT and NH herds. With an *in vitro* culture system of equine granulosa cells, Minervini *et al.* (2006) reported that ZEA and its metabolites (from 1×10^{-7} to 0.1 μM) induced apoptosis of granulosa cells, indicating that these mycotoxins could induce follicular atresia. Additionally, we previously reported that ZEA and its metabolites were detected in bovine follicular fluid and suggested that the concentrations of ZEA and its metabolites might be influenced by feeding conditions on farms (Takagi *et al.*, 2008). Therefore, although previous reports indicated that AMH concentrations were characteristic of each animal over a long-term period (Rico *et al.*, 2009, 2011), one possible reason for the significant difference in the ratio of AMH-positive animals between the 2 herds may be apoptosis of granulosa cells in

antral follicles induced by circulating ZEA and its metabolites, resulting in reduced AMH secretion from the granulosa cells into the blood. The remarkably high ZEA concentration in the straw collected at Day 58 in MYT herd compared with that in the straw collected at Day 50 in NM herd might have greatly affected to the significance of the number of AMH-valid cattle.

On the other hand, Weaver *et al.* (1986a, 1986b) reported that oral administration of 250 mg purified ZEA for 3 oestrous cycles caused an average conception rate of 62% compared with 87% in control heifers, and oral administration of 500 mg ZEA for 2 oestrous cycles resulted in normal serum P4 concentrations. We previously reported the *in vitro* effects of acute ZEA exposure on bovine oocytes by using *in vitro* maturation, *in vitro* fertilization (IVF), and *in vitro* culture systems in cattle, and suggested that a high concentration of ZEA might have a detrimental effect on the meiotic competence of bovine oocytes but does not affect fertilization and development after IVF (Takagi *et al.*, 2008). Additionally, we recently reported that natural ZEA contamination resulting in urine levels below the threshold value (i.e. below the maximal permissible urinary ZEA concentration) did not affect embryo production in Japanese Black and Holstein cows undergoing superovulation (Takagi *et al.*, 2013). Overall, the results of the present study on a ZEA-contaminated model herd together with the results of previous reports suggest one hypothesis that chronic ZEA contamination in cattle affects the population of antral follicles but does not harm the reproductive function of female cattle. However, our data must be interpreted carefully because of the small number of cattle herds assessed.

In conclusion, the results of our field trial conducted within a cattle herd naturally

contaminated with ZEA indicated that monitoring urinary concentrations of ZEA and its metabolites by LC/MS/MS is a useful tool for the objective evaluation of the effects of MA supplementation strategies in contaminated herds. Regarding MA supplementation of dietary feeds, at least for inhibition of the effects of ZEA and its metabolites, preventive effects on ZEA absorption from the intestine of cattle may be affected by the method of supplementation, i.e. mixing with or topdressing with dietary feeds, even for naturally occurring ZEA contamination levels. It was also confirmed that chronic ZEA exposure in cattle might not be directly reflected by the results of haematological and serum biochemical analysis. However, our results suggest that MA supplementation has beneficial effects on recovery of liver function as reflected by AST and GGT levels. Additionally, by measuring AMH and P4 concentrations in cattle herds with and without naturally occurring ZEA contamination, it was hypothesized that ZEA (acting as an endocrine disrupter) may affect the population status of antral follicles in bovine ovaries without affecting the ovulation status of female cattle. Further studies involving a greater number of cattle herds are required.

FIGURE LEGENDS

Figure 1.

Protocol for mycotoxin adsorbent (MA) supplementation in dietary feeds and sampling of blood and urine samples.

Figure 2.

Mean urinary zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), and total ZEA (ΣZEA; ZEA + α-ZOL + β-ZOL) concentrations during the experimental periods with or without mycotoxin adsorbent (MA; MX, MA was mixed with concentrates; TD, MA was topdressed on the concentrate; Cont, without MA supplementation) measured by the LC/MS/MS method in MYT herd (ZEA-contaminated herd).

a- b: significantly different ($P < 0.05$).

Note: The scaling in the different figures is different.

Figure 3.

Mean urinary zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), and total ZEA (ΣZEA; ZEA + α-ZOL + β-ZOL) concentrations during the experimental periods with or without mycotoxin adsorbent (MA; MX, MA was mixed with concentrates; TD, MA was topdressed on the concentrate; Cont, without MA supplementation) measured by the LC/MS/MS method in NM herd (control herd).

a- b: significantly different ($P < 0.05$).

Note: The scaling in the different figures is different.

Figure 1

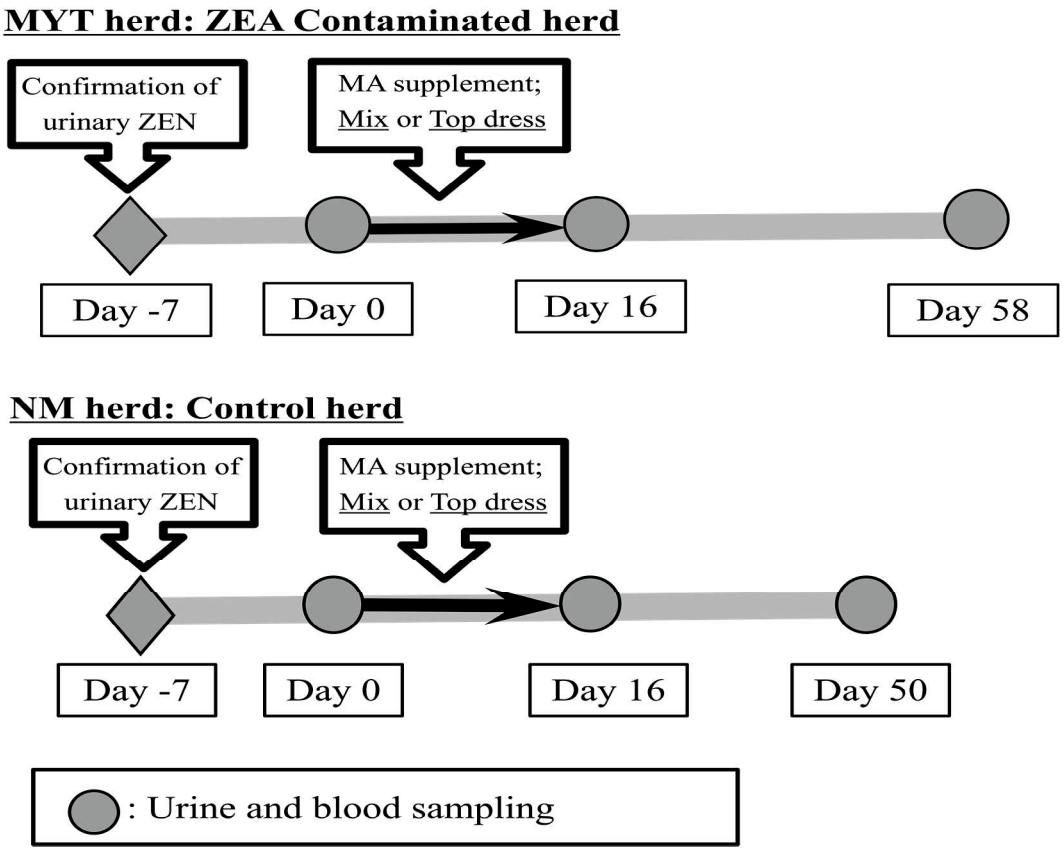
MYT herd: ZEA Contaminated herd
Confirmation of urinary ZEN
MA supplement; Mix or Top dress
Day -7
Day 0
Day 16
Day 58
NM herd: Control herd
Confirmation of urinary ZEN
MA supplement; Mix or Top dress
Day -7
Day 0
Day 16
Day 50
: Urine and blood sampling

Figure 2

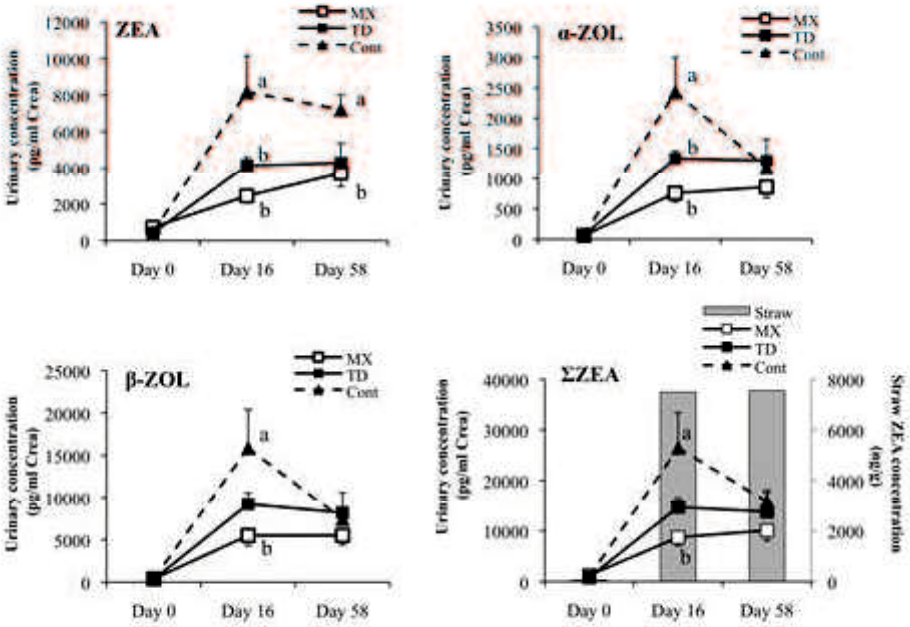

Figure 3

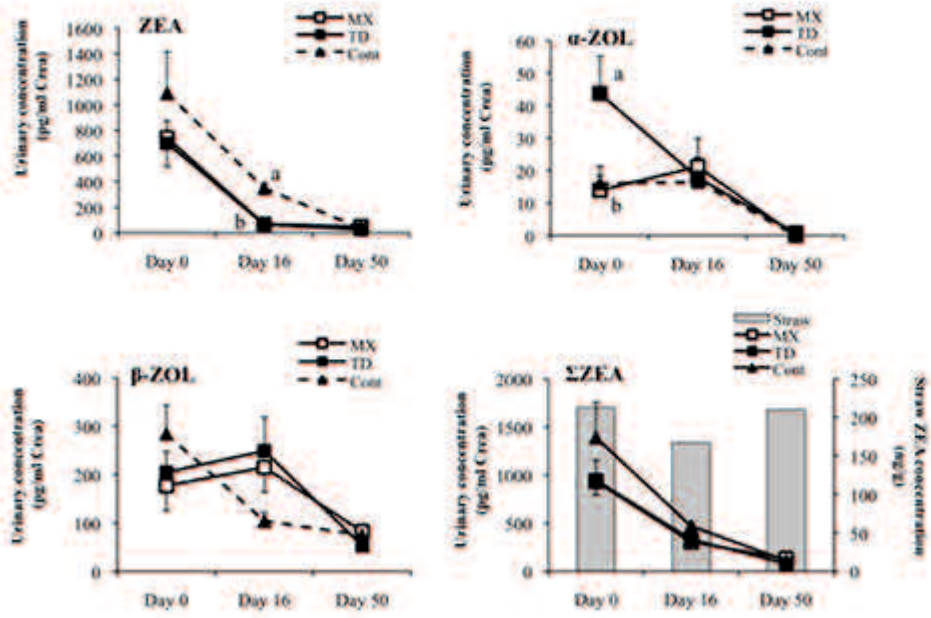

Table 1. Results of urinary ZEA concentrations measured by ELISA and ZEA concentrations of feed samples measured by high performance liquid chromatography (HPLC)

Group	Mean ± SEM (pg/mg of Crea)		
MYT herd; Contaminated herd	Day 0	Day 16	Day 58
MX (n=6)	1,958 ± 208[a]	30,975 ± 9,131	15,951 ± 4,178
TD (n=6)	906 ± 154[b]	17,985 ± 1,957	15,062 ± 3,550
Control (n=4)	2,688 ± 428[a]	52,720 ± 24,997	20,555 ± 2,808
Straw* (ng/g)	55	7493	7555
Concentrate* (ng/g)	138	137	110
NM herd; Control herd	Day 0	Day 16	Day 50
MX (n=6)	495 ± 29	467 ± 59	165 ± 35
TD (n=6)	491 ± 80	486 ± 73[a]	120 ± 11
Control (n=6)	676 ± 102	334 ±16[b]	168 ± 24
Straw* (ng/g)	213	167	210
Concentrate* (ng/g)	233	132	81

*: Measured by HPLC according to Emoto et al. (2008).
a-b: Values with different superscripts in the same column differ significantly ($P < 0.05$).

Table 2. Results of anti-Müllerian hormone (AMH) concentrations during the experimental periods with or without mycotoxin adsorbent measured in MYT herd (ZEA-highly contaminated) and NM herd (control)

			Number and AMH concentrations of cattle with AMH > 0.08 ng/mL**					
			Day 0		Day 16		Day 58 or Day 50	
Group	Treatment	No. of cattle*[1]	No (%) of valid*[2]	AMH[#] (ng/mL)	No. (%) of valid	AMH (ng/mL)	No. (%) of valid	AMH (ng/mL)
MYT herd	MX	6	4 (66.7)	0.13 ± 0.02	3 (50.0)	0.22 ± 0.04	2 (33.3)[a]	0.10, 0.11
(ZEA contaminated)	Control	5	3 (60.0)	0.30 ± 0.18	3 (60.0)	0.12 ± 0.01	1 (25.0)*[3a]	0.22
NM herd	MX	6	6 (100)	0.20 ± 0.06	4 (66.7)	0.33 ± 0.14	5 (83.3)	0.13 ± 0.02
(Control)	Control	5	4 (80.0)	0.25 ± 0.04	5 (100)	0.23 ± 0.09	5 (100)[b]	0.21 ± 0.03

MX: mycotoxin adsorbent was mixed with concentrates; Control: without supplementation of mycotoxin adsorbent.
*1: Because serum samples were not aailable for 1 animal, the number of animals in the control group of both MYT herd and 2 was 5 in the present study.
*2: Number of cattle whose AMH concentration was more than 0.08 ng/mL.
*3: Because 1 animal was excluded from the experiment at this sampling time because of sickness, the total number of this group was 4.
**: The detection limit of the ELISA assay used in the present study was 0.08 ng/mL.
#: Mean ± SEM of AMH concentration derived from the cattle whose AMH concentration was more than 0.08 ng/mL.
a-b: Significant difference ($P < 0.05$) within the same column.

Table 3. Results of haematological and serum biochemical analyses (mean±SEM) of the examined cattle in MYT herd

Parameters for protein metabolism					Parameters for energy metabolism				
	Group	Day 0	Day 16	Day 58		Group	Day 0	Day 16	Day 58
TP (g/dL)	MX	6.9 ± 0.2	7.1 ± 0.2	6.9 ± 0.2	FFA (μEq/L)	MX	222.3 ± 36.6	252.4 ± 54.7	228.5 ± 25.2
	TD	7.0 ± 0.1	6.9 ± 0.2	7.1 ± 0.1		TD	250.5 ± 43.7	307.1 ± 41.3	248.0 ± 39.4
	Cont	7.0 ± 0.1	7.1 ± 0.2	7.0 ± 0.4		Cont	191.2 ± 37.0	273.2 ± 42.9	236.2 ± 54.9
Alb (g/dL)	MX	3.6 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	T-Cho (mg/dL)	MX	167.2 ± 10.5	162.8 ± 9.8	172.5 ± 10.9
	TD	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1		TD	187.9 ± 15.5	173.5 ± 10.7	168.1 ± 13.0
	Cont	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.1		Cont	163.9 ± 9.1	169.2 ± 11.6	179.4 ± 14.5
AG	MX	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	Glu (mg/dL)	MX	68.0 ± 1.4	70.4 ± 2.1	74.8 ± 0.9
	TD	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1		TD	68.1 ± 2.8	74.9 ± 2.0	72.1 ± 2.5
	Cont	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2		Cont	65.8 ± 0.9	75.5 ± 1.8	73.6 ± 1.7
BUN (mg/dL)	MX	18.1 ± 0.9	18.3 ± 1.1	19.2 ± 0.6					
	TD	19.1 ± 1.4	20.2 ± 0.8	15.7 ± 0.9					
	Cont	18.1 ± 0.7	18.1 ± 0.9	18.2 ± 1.3					
Liver functions/damage					Liver metabolism				
AST (IU/L)	MX	85.2 ± 12.2	95.2 ± 11.8	74.2 ± 9.1[a]	IGF-1 (ng/mL)	MX	78.2 ± 11.3	93.0 ± 7.7	103.5 ± 15.8
	TD	82.4 ± 8.3	86.4 ± 9.1	134.6 ± 39.6		TD	70.4 ± 8.2	97.1 ± 11.5	69.2 ± 5.3
	Cont	105.3 ± 19.7	131.5 ± 45.9	161.7 ± 66.0[b]		Cont	63.8 ± 6.6	97.3 ± 8.7	80.4 ± 6.0
GGT (IU/L)	MX	29.7 ± 2.8	52.9 ± 17.5	36.3 ± 4.3[a]					
	TD	48.6 ± 10.9	40.0 ± 4.9	51.4 ± 10.4					
	Cont	64.1 ± 13.8	65.1 ± 9.8	70.7 ± 14.4[b]	Haematological analysis				
Minerals					RBC ($\times 10^6$/μL)	MX	788 ± 33	785 ± 9	805 ± 29
						TD	780 ± 46	769 ± 31	779 ± 29
						Cont	715 ± 24	746 ± 33	798 ± 19
Ca (mg/dL)	MX	9.6 ± 0.2	9.6 ± 0.2	9.4 ± 0.1	WBC (/μL)	MX	8,517 ± 586	9,350 ± 1,534	8,633 ± 924
	TD	9.4 ± 0.1	9.3 ± 0.1	9.2 ± 0.1		TD	8,400 ± 503	8,567 ± 629	10,720 ± 1,616
	Cont	9.7 ± 0.2	9.4 ± 0.1	9.4 ± 0.1		Cont	10,140 ± 1,113	9,450 ± 1,151	10,950 ± 1,235
Mg (mg/dL)	MX	1.9 ± 0.1	2.1 ± 0.1[a]	1.9 ± 0.1	Hb (g/dL)	MX	12.9 ± 0.4	12.6 ± 0.2	12.9 ± 0.4
	TD	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1		TD	13.0 ± 0.7	12.6 ± 0.3	12.8 ± 0.4
	Cont	2.0 ± 0.1	1.8 ± 0.1[b]	1.8 ± 0.1		Cont	12.3 ± 0.3	12.4 ± 0.5	13.1 ± 0.5
Fe (mg/dL)	MX	178.3 ± 15.6	199.8 ± 14.8	203.2 ± 8.4	PCV (%)	MX	35.7 ± 0.8	36.1 ± 0.8	37.1 ± 0.9
	TD	174.0 ± 16.1	210.7 ± 9.8	149.7 ± 28.3		TD	36.3 ± 1.6	35.7 ± 0.8	36.4 ± 1.0
	Cont	178.9 ± 12.6	203.4 ± 23.7	201.4 ± 34.8		Cont	34.8 ± 0.8	35.9 ± 1.2	37.0 ± 1.1

a-b: Values with different superscripts in the same column differ significantly ($P<0.05$).

TP: total protein, AG: albumin/globulin ratio, AST: aspartate aminotransferase, GGT: γ-glutamyltransferase, FFA: free fatty acid, T-Cho: total cholesterol, IGF-1: insulin-like growth factor-1, Hb: haemoglobin, PCV: packed cell volume

Table 4. Results of haematological and serum biochemical analyses (mean±SEM) of the examined cattle in NM herd

Parameters for protein metabolism					Parameters for energy metabolism				
	Group	Day 0	Day 16	Day 50		Group	Day 0	Day 16	Day 50
TP (g/dL)	MX	7.1 ± 0.2	7.5 ± 0.2	7.1 ± 0.1	FFA (μEq/L)	MX	174.6 ± 30.1	320.5 ± 54.5	124.0 ± 14.7
	TD	7.1 ± 0.2	7.4 ± 0.1	7.2 ± 0.2		TD	261.3 ± 80.9	198.8 ± 23.0[a]	116.4 ± 10.4
	Cont	7.1 ± 0.2	7.4 ± 0.2	7.1 ± 0.1		Cont	173.9 ± 26.0	352.6 ± 55.4[b]	101.5 ± 3.1
Alb (g/dL)	MX	3.5 ± 0.1	3.8 ± 0.1[a]	3.5 ± 0.1	T-Cho (mg/dL)	MX	143.0 ± 23.2	166.3 ± 27.9	158.5 ± 20.2
	TD	3.7 ± 0.1	3.5 ± 0.1[b]	3.5 ± 0.1		TD	151.8 ± 9.6	148.1 ± 4.8	163.4 ± 12.3
	Cont	3.5 ± 0.1	3.7 ± 0.1	3.5 ± 0.1		Cont	159.3 ± 13.7	169.6 ± 5.3	171.9 ± 10.3
AG	MX	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	Glu (mg/dL)	MX	65.0 ± 3.1	71.2 ± 1.7	65.4 ± 3.5[a]
	TD	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1		TD	68.7 ± 4.4	68.7 ± 3.5	70.4 ± 1.7
	Cont	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1		Cont	70.8 ± 5.1	68.0 ± 2.7	73.3 ± 3.3[b]
BUN (mg/dL)	MX	14.6 ± 1.0	17.0 ± 1.6	19.8 ± 1.2					
	TD	15.6 ± 1.0	19.4 ± 1.8	22.1 ± 1.2					
	Cont	14.6 ± 1.6	18.0 ± 1.7	21.5 ± 2.1					
Liver functions/damage					Liver metabolism				
AST (IU/L)	MX	86.8 ± 8.4	92.4 ± 10.9	108.1 ± 18.3	IGF-1 (ng/mL)	MX	106.6 ± 25.5	93.7 ± 34.3	94.9 ± 29.7
	TD	106.3 ± 14.1	138.9 ± 11.3	101.2 ± 6.9		TD	123.3 ± 20.6	82.3 ± 8.9	94.2 ± 10.7
	Cont	97.1 ± 10.5	125.9 ± 34.1	91.6 ± 7.1		Cont	81.3 ± 8.9	87.0 ± 9.5	80.1 ± 8.5
GGT (IU/L)	MX	42.0 ± 7.5	34.0 ± 5.8	27.7 ± 1.4					
	TD	76.8 ± 30.2	50.9 ± 8.2	47.6 ± 8.7	Haematological analysis				
	Cont	49.5 ± 8.7	53.7 ± 15.9	39.1 ± 6.1					
Minerals					RBC ($\times 10^6$/μL)	MX	839 ± 53	919 ± 44[a]	806 ± 39
						TD	840 ± 19	845 ± 15	814 ± 16
						Cont	773 ± 30	813 ± 27[b]	736 ± 44
Ca (mg/dL)	MX	9.7 ± 0.2	9.5 ± 0.1	9.3 ± 0.2	WBC (/μL)	MX	10,100 ± 755	10,967 ± 1,350	10,983 ± 1,069
	TD	9.4 ± 0.2	9.4 ± 0.1	9.3 ± 0.1		TD	11,317 ± 1,048	10,217 ± 1,047	8,400 ± 574
	Cont	9.7 ± 0.1	9.2 ± 0.1	9.3 ± 0.1		Cont	9,900 ± 1,591	9,483 ± 523	8,640 ± 275
Mg (mg/dL)	MX	2.1 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	Hb (g/dL)	MX	13.3 ± 0.3	14.6 ± 0.4	12.4 ± 0.5
	TD	2.1 ± 0.2	1.8 ± 0.1	2.0 ± 0.1		TD	13.9 ± 0.5	13.8 ± 0.4	13.0 ± 0.4
	Cont	2.2 ± 0.1	1.9 ± 0.1	2.0 ± 0.1		Cont	13.3 ± 0.5	14.0 ± 0.4	12.5 ± 0.7
Fe (mg/dL)	MX	141.7 ± 2.1	150.4 ± 13.7	149.3 ± 14.3[a]	PCV (%)	MX	37.7 ± 1.1	41.8 ± 1.2	36.1 ± 1.5
	TD	146.6 ± 24.3	136.3 ± 27.8	206.8 ± 11.3[b]		TD	39.4 ± 1.3	39.4 ± 1.1	37.1 ± 0.9
	Cont	169.2 ± 21.0	169.9 ± 10.7	197.3 ± 17.0		Cont	37.2 ± 1.0	40.0 ± 1.0	35.3 ± 1.8

a-b: Values with different superscripts in the same column differ significantly ($P<0.05$).

Chapter 2

Effects of dietary contamination by zearalenone and its metabolites on serum anti-Müllerian hormone: impact on the reproductive performance of breeding cows

ABSTRACT

We investigated the effects of *in vivo* exposure to low zearalenone levels on the anti-Müllerian hormone endocrine levels and the reproductive performance of cattle. Urine and blood samples and reproductive records were collected from two Japanese Black breeding female cattle herds with dietary zearalenone contamination below the threshold levels (< 1 ppm) at 30 d after calving. Urinary zearalenone, α-zearalenol, and β-zearalenol concentrations were measured by chromatography–tandem mass spectrometry, and serum anti-Müllerian hormone concentrations were determined along with serum biochemical parameters. Urinary concentrations of α- zearalenol were significantly higher ($p < 0.05$) in cattle in Herd 1 than in cattle in Herd 2, reflecting the different amounts of zearalenone in the diet of the two herds. Although the number of 5-mm and 10-mm follicles of the herds and their fertility after artificial insemination were similar, the serum anti-Müllerian hormone concentrations in Herds 1 and 2 were 438.9 ± 48.6 pg/mL and 618.9 ± 80.0 pg/mL, respectively, with a trend toward a significant difference ($p = 0.053$), which may indicate differences in the antral follicle populations between herds. Thus, zearalenone intake from dietary feed, even when below the threshold zearalenone contamination level permitted in Japan, may affect the ovarian antral follicle populations, but not the fertility, of postpartum cows.

INTRODUCTION

Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species in plants, including pasture grasses. Because its lactone ring resembles many of the structural features of the aromatic rings of estradiol and apparently fits into the binding pocket of the mammalian estrogen receptor, ZEA and its metabolites exhibit distinct estrogenic and anabolic properties that affect the reproductive systems of several animal species (Kleinova et al. 2002; Fink-Gremmels and Malekinejad, 2007; Minervini and Dell'Aquila 2008). Thus, these substances are generally classified as endocrine disrupters. Several *in vivo* trials and *in vitro* assays have been conducted thus far to determine the effects of ZEA and its metabolites on the reproductive function of cattle; although the results largely support the above hypothesis, some contradictions remain. In an *in vitro* culture system of equine granulosa cells, ZEA and its metabolites (from 1×10^{-7} to 0.1 μM) induced granulosa cell apoptosis (Minervini et al. 2006). A recent study confirmed that ZEA induces apoptosis and necrosis of granulosa cells in a dose-dependent manner via a caspase-3- and caspase-9-dependent mitochondrial pathway in a mouse model (Zhu et al. 2012). These studies suggest that ZEA and its metabolites induce ovarian follicular atresia.

Anti-Müllerian hormone (AMH) is a glycoprotein exclusively produced by the granulosa cells of growing ovarian follicles in the adult female (Vigier et al. 1984; Monniaux et al. 2013). AMH is a key factor that inhibits the recruitment of primordial follicles into the pool of growing follicles and decreases the responsiveness of growing follicles to follicle-stimulating hormone (Durlinger et al. 2002; Visser and Themmen 2014). Currently, AMH is considered to be the best endocrine marker of the population of small antral gonadotropin-responsive follicles in cows (Rico et al. 2009; Ireland et al. 2011; Monniaux et al. 2014). On the other hand, we previously reported that ZEA and its metabolites can be

detected in bovine follicular fluid, and our findings suggested that the feeding conditions on different cattle farms influence the concentrations of ZEA and its metabolites in the animals' follicular fluid (Takagi et al. 2008). Therefore, circulating ZEA and its metabolites might modulate follicular development by inducing apoptosis of granulosa cells in the antral follicles, leading to reduced AMH secretion from the granulosa cells of atretic antral follicles. Recently, a cattle herd raised for fattening purposes with significantly higher urinary ZEA concentrations than other herds due to the intake of ZEA-contaminated rice straw has been characterized as a useful model for studying the effects of ZEA contamination under farming conditions (Takagi et al. 2011; Hasunuma et al. 2012). The comparison of urine and serum samples of some animals from this highly contaminated herd with samples from a control herd given the same feed except for the rice straw suggested that the contamination of dietary feed with ZEA and its metabolites affects AMH concentration in the serum of cattle herds (Fushimi et al. 2014). However, the effect of different levels of ZEA contamination in feed on AMH endocrine levels has not yet been established.

The objective of this study was to investigate the effects of *in vivo* exposure to low ZEA contamination levels (below the threshold levels mandated in Japan) on the serum AMH concentration of breeding cattle herds, with regard to their reproductive performance.

MATERIALS AND METHODS

Japanese Black cattle herds and sample collection

Two herds (Herd 1 and Herd 2) of Japanese Black cows maintained for breeding in Kagoshima Prefecture, Japan, were included in this experiment. Herds 1 and 2 consisted of 90 and 80 adult cows, respectively. Cows in Herd 1 were fed fescue and oats hay as roughage, along with a purchased concentrate, whereas cows in Herd 2 were fed orchard grass and whole-crop silage (WCS) as roughage, along with a purchased concentrate. In both herds, the roughages and concentrates were stored in feed sheds and silos, respectively, at ambient temperature.

Before starting the experiment, the ZEA level in the dietary feed of both herds was measured via liquid chromatography–tandem mass spectrometry (LC/MS/MS), as previously described (Fushimi et al. 2014). The concentrations of ZEA in the fescue grass, oats hay, and concentrates fed to cows in Herd 1 were < 0.01, 0.1, and < 0.01 ppm, respectively. The ZEA concentrations in orchard grass, WCS, and concentrates fed to cows in Herd 2 were 0.01, < 0.01, and 0.05 ppm, respectively. These findings confirmed that the ZEA contamination levels of the dietary feeds were below the threshold levels applied in Japan (< 1.0 ppm) in both herds.

Urine and blood samples were obtained from the cows of both herds exactly 30 d after parturition, when the postpartum evaluation of reproductive organs was conducted. At 2 h after the morning feed, spontaneous urine samples were collected from the animals by massaging the pudendum, and blood samples were collected from the jugular vein in silicone-coated tubes. Concomitant with these samplings, ultrasound (US) scanning of both ovaries and the uterus were conducted as a flesh check using a liner array US scanner equipped with a 5 MHz rectal probe, as previously reported (Takagi et al. 2005). The ovaries

were monitored simultaneously in several planes, and all follicles > 5 mm in diameter and corpora lutea (CL) > 5 mm in diameter were identified. In addition, the uterus was monitored to confirm postpartum recovery to normal uterine conditions. Reproductive performance data were also obtained from each herd record afterwards, when all of the examined cows became pregnant. For each herd, the data included the day of the first artificial insemination (AI; performed at the time of the first heat detection after calving), the number of AIs needed to establish pregnancy, and the day-open period (number of days between calving and the AI that was followed by pregnancy).

All samples were immediately placed in a box on ice for cooling and protection from light and were transported to the laboratory. After centrifugation at 500 × *g* for 10 min at room temperature, the urine and blood samples were frozen at −30°C until analysis of ZEA and creatinine concentrations in the urine samples and biochemical parameters in the serum samples. Urinary concentrations of ZEA, α-ZOL, and β-ZOL were measured using LC/MS/MS. To assess the metabolic profiles of the cattle from both herds, serum biochemical analysis was performed (Labospect 7180 autoanalyzer, Hitachi, Tokyo, Japan) to determine the following parameters, according to our previous report (Watanabe et al. 2013): blood glucose, free fatty acids (FFA), and total cholesterol (TC) for evaluation of energy metabolism; total protein (TP), serum albumin (Alb), albumin/globulin (A:G) ratio, and blood urea nitrogen (BUN) for protein metabolism; serum aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) for liver function; and calcium (Ca), magnesium (Mg), and inorganic phosphorus (iP) for minerals. Additionally, to evaluate hepatic metabolism, the concentration of insulin-like growth factor 1 (IGF1) was determined in the serum using an enzyme immunoassay with the biotin-streptavidin amplification technique (Kawashima et al. 2007).

Methods of urine sample analysis

All urine samples in this experiment were analyzed by LC/MS/MS, as described in our previous report (Takagi et al. 2011), and confirmed the ZEA, α-ZOL, and β-ZOL concentrations previously reported. The LC/MS/MS analyses were performed on an API 2000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface and a 1200 Infinity Series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA). The detection limits for ZEA, α-ZOL, and β-ZOL were 0.04 ng/mL, 0.05 ng/mL, and 0.05 ng/mL, respectively, and the mean recovery rates for ZEA, α-ZOL, and β-ZOL were 116%, 121%, and 56%, respectively. The urine creatinine concentrations were determined using a commercial kit (Sikarikit-S CRE, Kanto Chemical, Tokyo, Japan) according to the manufacturer's instructions and were measured with a 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan). All urinary concentrations were expressed in a ratio to creatinine (pg/mg creatinine), as described previously (Takagi et al. 2011).

Serum AMH analysis

The AMH concentrations of all stocked serum samples were measured by using a commercially available AMH Gen II ELISA kit (Beckman Coulter, Inc., Brea, CA, USA) according to the method of Rico et al. (2012). AMH concentrations were determined in 50 μl of undiluted serum. The sensitivity of this assay was 15 pg/mL, and the intra-assay coefficients of variation were 3% and 5% for quality-controlled plasma samples containing 80 pg/mL and 800 pg/mL of AMH, respectively.

Statistical analysis

The results for urinary ZEA, α-ZOL, and β-ZOL, total ZEA (ΣZEA; ZEA + α-ZOL + β-ZOL), serum AMH concentrations, and all biochemical parameters and reproductive

records are expressed as the mean ± SEM. The analyses of urine, serum, and reproductive records of Herd 1 and Herd 2 were compared using the student's t-test with the Welch correction when the variances differed. P values < 0.05 were considered to indicate a statistically significant difference, while P values < 0.1 were considered to indicate a trend toward significance.

RESULTS

The cows recruited from Herd 1 were younger than the cows recruited from Herd 2 (p = 0.04, Table 1). The urinary ZEA, α-ZOL, β-ZOL, and ΣZEA concentrations, mean serum AMH concentration, ovarian status, and reproductive performance of Herds 1 and 2 are shown in Table 1. Significant differences in urinary α-ZOL (p = 0.02) and tendencies toward significant differences were observed in urinary ZEA (p = 0.09) and ΣZEA (p = 0.08) between Herds 1 and 2, suggesting that the ZEA amount in the dietary feed was significantly higher in Herd 1 than in Herd 2. On the other hand, the serum AMH concentration of cows in Herd 1 tended to be significantly lower (p = 0.053) than that of the cows in Herd 2. Regarding ovarian status, no significant differences in the number of follicles between 5 mm and 10 mm in diameter were observed between Herds 1 and 2. The postpartum day of the first AI tended to be earlier and the day-open period shorter in Herd 1 (p = 0.06 and p = 0.08, respectively), but the numbers of AIs needed to establish pregnancy in both herds were not different.

The results of the mean serum biochemical analyses from Herd 1 and Herd 2 are shown in Table 2. Significant differences were observed between the two herds in BUN (p < 0.01) and TC (p < 0.0001) concentrations. However, no significant differences were observed for the other parameters within their normal ranges in both herds.

DISCUSSION

We previously described a cattle herd raised for fattening purposes with significantly higher urinary ZEA concentrations than other herds (Takagi et al. 2011) because of the intake of contaminated rice straw containing > 8 ppm ZEA (Hasunuma et al. 2012). Recently, in a preliminary study, we reported the possible effects of *in vivo* exposure to this level of contamination of ZEA and its metabolites on the population of antral follicles, as assessed by significantly lower serum AMH levels measured in the stocked serum samples from the above-mentioned ZEA-contaminated herd (Fushimi et al. 2014). Therefore, the objective of this study was to evaluate the relationship between urinary ZEA, including its metabolites, and serum AMH concentrations in commercial breeding cattle herds exposed to lower ZEA contamination levels. The results suggest that the ZEA contamination levels in the dietary feed of cattle herds decreased the serum AMH concentrations of the female cattle, even when the ZEA contamination in the dietary feed is under the prescribed threshold ZEA level.

ZEA and its metabolites exhibit distinct estrogenic properties that affect the reproductive system of several animal species, especially pigs (Kleinova et al. 2002; Fink-Gremmels and Malekinejad 2007; Minervini and Dell'Aquila 2008). On the other hand, clinical signs of hyperestrogenism are not frequently observed in ruminating cows, and only following the ingestion of highly contaminated silage or long-term exposure to contaminated feed materials (Weaver et al. 1986a,b; Fink-Gremmels 2008). Indeed, we previously reported the *in vitro* effects of acute ZEA exposure on bovine oocytes by using *in vitro* maturation, *in vitro* fertilization (IVF), and *in vitro* culture systems in cattle, and we found that a high ZEA concentration (> 1 ppm in the culture medium) might have a detrimental effect on the meiotic competence of bovine oocytes, but does not affect fertilization and development after IVF (Takagi et al. 2008). Additionally, we recently reported that natural-feed ZEA contamination

levels below the threshold value (i.e., below the maximum permissible ZEA concentration in Japan) did not affect embryo production in Japanese Black and Holstein cows undergoing superovulation (Takagi et al. 2013). Therefore, the results of the present study suggest that ZEA intake/exposure has a novel significant effect within cattle ovaries that does not produce any general clinical signs of hyperestrogenism.

Cows in the postpartum period may be particularly susceptible to environmental contaminants because the negative energy balance is exacerbated by the presence of molds and mycotoxins in the diet (Fink-Gremmels 2008). Thus, in the present study, we obtained both blood and urine samples at 30 d after calving for conducting (1) the flesh check (first postpartum examinations to check the recovery of the reproductive organs) to adjust the postpartum nutritional/metabolic levels of each herd, and (2) the serum biochemical analysis, to objectively monitor the metabolic status of the herds.

Under the present experimental conditions, the results regarding ovarian status and postpartum reproductive parameters (day of first AI, number of AIs, and day-open period), together with the urinary ZEA, indicate that significant differences in the ZEA levels in dietary feed do not affect cattle reproductive performance, at least under normally controlled breeding-farm conditions, as reported by others (Weaver et al. 1986a,b; Fink-Gremmels 2008). The cows in Herd 2 were significantly older than the cows in Herd 1 at the time of sampling. In the cow, as in other mammals, aging is associated with lower fertility (Erickson et al. 1976; Malhi et al. 2005). The tendency for the cows in Herd 2 to have a delay in the first AI and a longer day-open period might be related to the age difference between the herds. However, it cannot explain the tendency of the cows in Herd 2 to have higher serum AMH concentrations. In fact, older cows are expected to have lower serum AMH concentrations, as the ovarian reserve of follicles are known to be depleted in aging mammals (Dewailly et al. 2014; Monniaux et al. 2014). Moreover, older cows have been shown to have fewer small antral

follicles (Cushman et al. 2009) and decreased responses to superovulation treatment (Malhi et al. 2008).

We propose that the tendency for cows in Herd 1 to have lower serum AMH concentrations than those in Herd 2 is related to their higher ZEA dietary contamination level, as shown by the urinary concentrations of ZEA and its metabolites. In female cattle, ovarian AMH expression is restricted to the granulosa cells of healthy small antral follicles and is strongly decreased when the follicles enter atresia (Rico et al. 2009, 2011). A previously study demonstrated in an *in vitro* culture system that ZEA and its metabolites can induce the apoptosis of granulosa cells in a dose-dependent manner via a caspase-3- and caspase-9-dependent mitochondrial pathway (Zhu et al. 2012), resulting in follicular atresia. Altogether, these observations suggest that the difference in AMH endocrine levels between herds reflects a difference in the atresia rate of the populations of small antral ovarian follicles, which are affected by different ZEA contamination levels. In any case, ZEA, at least at low dietary contamination levels, would not modulate terminal follicular development and ovulation, and would not affect the fertility of cows with low contamination levels.

However, other factors noted in Herd 1 and 2 may have greatly affected the reproductive performance of each herd. These factors mainly included postpartum feeding and reproductive management and the nutritional level of the dietary feed, as demonstrated by significant differences in BUN (reflecting protein metabolism) and TC (reflecting energy metabolism) between the two herds. Moreover, the consensus is that most contaminated feed samples contain more than one mycotoxin (Monbaliu et al. 2010; van Pamel et al. 2011; Streit et al. 2012). Under field conditions, cows are exposed to complex mixtures of toxins originating from roughage and concentrates; the detoxifying capacity of the rumen microflora is exhausted, and unchanged mycotoxins are absorbed via the duodenum, resulting in an unexpectedly high internal challenge (Fink-Gremmels 2008). Further studies are required to

establish a larger database derived from a greater number of cattle herds.

In conclusion, our study of Japanese Black breeding herds fed with ZEA-controlled feed management (below the permitted threshold levels) suggests that a relationship exists between the contamination level of ZEA and its metabolites and the serum AMH concentration, even when ZEA dietary contamination is low. Further studies are required, and these studies should include *in vivo* trials with greater numbers of cattle herds as well as *in vitro* co-exposure experiments in isolated bovine antral follicle cultures with both ZEA and its metabolites and other mycotoxins.

Table 1.

Mean concentrations of (1) urinary ZEA (± SEM) and its metabolites and (2) serum anti-Müllerian hormone (AMH) related to the reproductive performance of the two Japanese Black breeding cattle herds examined.

	Herd 1	Herd 2
No. Cattle Examined (mean age ± SEM)	n = 17 (5.4 ± 0.7)[a]	n = 13 (8.0 ± 1.0)[b]
ZEA concentration in dietary feeds (ppm)	Fescue < 0.01, oats hay 0.1, concentrates < 0.01	Orchard grass 0.01, WCS < 0.01, concentrates 0.05
ZEA (pg/mg creatinine)	235.3 ± 103.2[c]	45.6 ± 14.2[d]
α-ZOL (pg/mg creatinine)	133.2 ± 39.9[a]	16.5 ± 5.6[b]
β-ZOL (pg/mg creatinine)	341.2 ± 84.9	220.2 ± 60.4
Σ ZEA (pg/mg creatinine)	709.7 ± 224.0[c]	282.3 ± 63.0[d]
AMH (pg/mL)	438.9 ± 48.6[c]	618.9 ± 80.0[d]
No. follicles	5 mm, 4.1 ± 0.9; 10 mm, 0.8 ± 0.1	5 mm, 3.0 ± 0.5; 10 mm, 0.7 ± 0.2
Cattle with CL	11 (64.7%)	7 (53.8%)
Day of first AI	50.2 ± 4.0[c]	77.1 ± 12.3 (n = 10)[d]*
No. of AIs	1.9 ± 0.4	2.4 ± 0.4 (n = 10)*
Day-open period	82.2 ± 16.1[c]	128.3 ± 17.5 (n = 10)[d]*

*: Only 10 animals in Herd 2 were examined because three animals were excluded before their first AI.

[a], [b]: Significant difference ($p < 0.05$) between columns.

[c], [d]: Trend toward significant difference ($0.05 < p < 0.1$) between columns.

Table 2.

Results of serum biochemical analyses (mean ± SEM) of the two Japanese Black breeding cattle herds examined.

	Herd 1	Herd 2
No. Cattle Examined	n = 17	n = 13
Energy metabolism		
Blood glucose (mg/dL)	45.1 ± 2.9	46.5 ± 3.4
Free fatty acids (μEq/L)	107.0 ± 12.0	101.3 ± 9.4
Total cholesterol (mg/dL)	119.9 ± 4.7[a]	89.0 ± 3.3[b]
Protein metabolism		
Total protein (g/dL)	6.4 ± 0.1	6.6 ± 0.1
Serum albumin (g/dL)	3.1 ± 0.1	3.1 ± 0.1
A:G ratio	0.94 ± 0.04	0.89 ± 0.04
BUN (mg/dL)	8.0 ± 0.3[a]	10.3 ± 0.7[b]
Liver function		
AST (IU/L)	63.0 ± 2.2	63.6 ± 3.0
GGT (IU/L)	14.3 ± 1.4	24.9 ± 8.5
Minerals		
Ca (mg/dL)	9.1 ± 0.1	9.0 ± 0.1
Mg (mg/dL)	1.94 ± 0.04	1.91 ± 0.04
iP (mg/dL)	5.6 ± 0.2	5.2 ± 0.3
Liver metabolism		
IGF1 (ng/mL)	64.6 ± 4.1	72.3 ± 7.6

[a], [b]: Significant difference ($p < 0.01$) between columns.

Chapter 3

Measurement of sterigmatocystin concentrations in urine for monitoring the contamination of cattle feed

ABSTRACT

This study aimed (1) to determine the levels of the fungal toxin sterigmatocystin (STC) in the feed and urine of cattle and (2) to evaluate the effects of supplementing the feed with a mycotoxin adsorbent (MA) on STC concentrations in urine. Two herds of female Japanese Black cattle were used in this study. The cattle in each herd were fed a standard ration containing rice straw from different sources and a standard concentrate; two groups of cattle from each herd (n = 6 per group) received the commercial MA, mixed with the concentrate or given as top-dressing, whereas a third group received no supplement and served as control. Urine and feed samples were collected at various time points throughout the experiment. STC concentrations were measured using liquid chromatography-tandem mass spectrometry. STC concentrations in straw were higher in herd 1 (range: 0.15–0.24 mg/kg DM) than in herd 2 (range: <0.01–0.06 mg/kg DM). In Herd 1, STC concentrations in urine significantly declined 2 wks after replacing the contaminated feed, whereas MA supplementation had no effect. In conclusion, mycotoxins in urine samples are useful biological markers for monitoring the systemic exposure of cattle to multiple mycotoxins, as well as evaluating the effectiveness of interventions.

INTRODUCTION

Sterigmatocystin (STC) is a fungal secondary metabolite produced by fungi of the genera *Aspergillus* and *Penicillium*. STC is the end product of a biosynthetic pathway in some fungal species such as *A. versicolor* and *A. nidulans*, but is also a well-known precursor of aflatoxin B1 synthesis in various other fungal species. (Hsieh et al., 1973; Wilkinson et al., 2004; Versilovskis et al., 2010). STC has been shown to be genotoxic and potentially carcinogenic in studies with experimental animals (EFSA, 2013), and exerts teratogenic effects at higher exposure levels (Kusunoki et al., 2011). The European Food Safety Authority (EFSA) recently concluded that despite its potential carcinogenic effects, STC is of minor concern to human health in Europe due to its limited prevalence in European food commodities and subsequently a high margin of exposure (MoE) index. However, at the same time, the EFSA concluded that there were not sufficient data to draw conclusions about animal exposure and potential adverse health effects in animals, including cattle (EFSA, 2013). STC is frequently reported as a contaminant in feeds (e.g., grains, maize, and rice straw), but data on its adverse health effects in cattle is scarce and hence maximum exposure limits have not been established.

Exposure to mycotoxins is usually assessed by analyzing feed materials of total mixed rations. An alternative approach is the measurement of toxin concentrations in biological samples (such as urine) that reflect the individual exposure levels (Prelusky et al., 1989; Solfrizzo et al., 2011). Previously, we established a urine monitoring system for the mycotoxin zearalenone (ZEN) and its metabolites in cattle (Takagi et al., 2011; Hasunuma et al., 2012). In this study, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) of urine samples collected from these cattle herds to investigate the potential co-exposure to STC. We also used this approach to assess the efficacy of a commercial

mycotoxin adsorbent (MA).

MATERIALS AND METHODS

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

Chemicals and solvents

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/arylsulfatase solution 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).

Cattle herds and sample collection

Two herds of female Japanese Black cattle kept for fattening in Kagoshima Prefecture, Japan, were included in this study, as described more detail in our previous work (Hasunuma et al., 2012). Herds 1 and 2 were fed from the same lot of concentrate purchased from the same company, but they received different straw (Herd 1: purchased, Herd 2: self-produced). Three groups of six cattle of the same age (23 mo) and similar body weight (550–600 kg) were randomly selected from each of Herds 1 and 2 and divided into three treatments that differed in feed supplementation as follows: MA1, fed MA mixed with concentrate; MA2, fed MA applied as topdressing on concentrate; and control, no MA supplementation. The supplemented MA (Mycofix®Plus3.E, Biomin) was a commercially available product comprising minerals and biological constituents, including enzymes, yeast cell wall, clay, and plant extracts (Rank et al., 2011). The maximum daily dose of MA recommended by the

supplier (50 g) was provided in two 25-g doses per day for a 16-d period.

At 2 h after the morning feeding, spontaneous urine samples were collected from the animals by massaging the perineum. This activity was performed at the start of feed supplementation (Day 0), on the final day of supplementation (Day 16), and on the final day of the observation period (Day 58 for Herd 1; Day 50 for Herd 2). In addition, samples of rice straw and feed concentrate (approximately 1 kg each) were obtained from both the herds to measure STC concentrations in the feed. The protocol for MA supplementation and all sampling procedures are summarized in Figure 2. All samples were immediately placed in a cooler containing ice for protection from light and were transported to the laboratory. Urine samples were centrifuged at 500 ×*g* for 10 min at room temperature to remove the debris. The urine and feed samples were frozen at −30°C until the analysis of STC and creatinine.

Analysis of STC in the feed

STC concentrations were measured in straw and concentrate by using an API 3200 LC-MS/MS (Applied Biosystems) equipped with an ESI interface and a Prominence HPLC system (Shimadzu Corp., Kyoto, Japan), according to FAMIC (2012) [18] at Shokukanken Inc., Gunnma, Japan. In brief, representative samples of stored straw (2 g) and concentrate (10 g) were homogenized and chopped into small pieces. Each sample was placed in a sample tube, to which 20 mL of 84% acetonitrile was added. The tubes were shaken for 1 h and centrifuged for 10 min at 500 ×*g* at room temperature. The supernatant (10 mL) was loaded onto a MultiSep 226 Aflazon+ Multifunctional column (Romer Labs, MO, USA). Subsequently, 1 mL of the eluent was mixed with 1 mL acetic acid (1 + 100) and centrifuged for 5 min at 500 ×*g*.

Next, 10 μL supernatant was injected into the LC-MS/MS system under the following conditions: column, Gemini C18 (2 mm × 50 mm, 3 μm); oven temperature, 40°C; eluent

flow, 200 μL/min; and solvent, 0.1% aqueous formic acid (A) + 0.1% formic acid in methanol (B). An ESI probe was used in the positive mode for STC analysis. The ESI conditions were as follows: curtain gas, 20 psi; ion-spray voltage, 4,500 V; turbo temperature, 600°C; collision energy, 49.0 eV; declustering potential, 55.0 V; and entrance potential, 10.0 V. The detection limit for each analyte was 0.01 mg/kg. The mean STC recovery rates were 90.5–93.5%.

Analysis of STC and creatinine in the urine

Concentrations of STC were determined using LC-MS/MS by using an API 2000 system (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization (ESI) interface and a HPLC system (1200 Series; Agilent Technologies, Santa Clara, CA). In brief, the urine samples (0.5 mL) were 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 h at 37°C. After incubation, the solution was loaded onto a C18 solid-phase extraction (SPE) column (Strata, Phenomenex, Torrance, CA) that had been preconditioned with 3 mL 100% MeOH and 2 mL Tris buffer. An additional 2 mL Tris buffer and 3 mL 40% MeOH were added. After elution with approximately 1 mL 80% MeOH, the volume was adjusted to exactly 1 mL.

Next, 20 μL of solution was injected into the LC-MS/MS system. Chromatographic separation was performed on an Inertsil ODS-3 column (4.6 i.d. × 100 mm, 5 μm; GL Sciences, Tokyo, Japan) at 40°C. 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 measurement time was 15 min. A multiple reaction monitoring system was used to switch STC (*m*/*z* 325.0–281.0) to the positive ion mode. Instrumental parameters were optimized for STC measurement by analyzing the corresponding standard solution at a flow rate of 10 μL/min by using a syringe pump integrated into the API-2000 MS. The electrospray

conditions were as follows: curtain gas, 40 psi; ion-spray voltage, 5,500 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. The mean recovery rate was between 85% and 120% in our assay. Representative chromatograms from the LC-MS/MS assay are shown in Figure 3.

Concentrations of creatinine in the urine were determined using a commercial kit (Sikarikit-S CRE; Kanto Chemical) according to the manufacturer's instructions and measured using a clinical autoanalyzer (7700 Clinical Analyzer; Hitachi High-Tech, Tokyo, Japan). STC concentrations in the urine were expressed as a ratio to creatinine (pg STC/mg creatinine), as described previously (Miles et al., 1996; Padilla et al., 2007).

Fungal cultures

Fungal samples were cultured directly from the samples of infected rice straw selected from the two herds. Each straw sample (5-mm^2 fragment) was soaked in 1% sodium hypochlorite for 1 min, rinsed in sterile water three times, plated on Czapek Dox agar (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, 3% sucrose, w/v), and incubated at 25°C in the dark for 7 d. Fungal colonies that emerged on the straw were observed, and the fungi were identified macroscopically.

Statistical analysis

Concentrations of STC in the urine collected from each herd during the three sampling events were compared among the three groups (MA1, MA2, control) by using one-way analysis of variance (ANOVA) followed by a post-hoc test performed the StatView program (Abac Concepts, Inc., Berkeley, CA) to evaluate the effects of MA supplementation. The significance threshold was set at $P < 0.05$. Results for STC concentrations are expressed as

means ± SE.

RESULTS

Urinary metabolites of STC

To establish an analytical method for the quantification of STC in urine, preliminary trials for STC extraction were conducted, including a comparison of a direct extraction of STC and a pre-incubation of the urine samples with β-glucuronidase/arylsulfatase. The results of these analyses are presented in Table 1 and clearly indicated that the major urinary metabolite of STC is the conjugate with glucuronic acid. Therefore, in all forthcoming analyses, the urine samples were pre-incubated with β-glucuronidase/arylsulfatase before the extraction of STC.

Concentrations of STC in urine and feed samples

In the current study, three groups of six cattle of the same age (23 months) and similar body weight (550–600 kg) were randomly selected from Herds 1 and 2 and divided into three treatments that differed in feed supplementation as follows: MA1, fed MA mixed with concentrate; MA2, fed MA applied as topdressing on the concentrate; and control without MA supplementation. Clinical signs of toxicity associated to mycotoxin exposure had not been reported by the owners of the animals used in the trial, with the exception of chronic diarrhea in Herd 1 cattle due to unknown causes. The STC concentrations in rice straw and concentrate are shown in Table 2 together with the urinary STC concentrations. The STC concentrations in straw were two- to five-fold higher in Herd 1 than in Herd 2 at each sampling time. However, the STC concentrations in all samples of dietary concentrate from both the herds were below the detection limits of the assay (0.01 mg/kg). STC concentrations in the urine of Herd 1 control group were significantly higher ($P < 0.05$) than those in Herd 2 control group on Day 0 and the third sampling day (Day 56 in Herd 1 and Day 50 in Herd 2).

In Herd 1, there were significant differences in urine STC concentrations between MA2 and the control group on Day 58 ($P < 0.05$), and between MA1 and the other two groups on Day 72 ($P < 0.05$).

Fungal cultures

The results for fungal cultures from the collected straw are shown in Figure 1. Fungal colonies from Herd 1 on Day 0 were almost exclusively *Aspergillus niger*, but also included a small colony of *Fusarium graminearum* species complex, which was expected, because Fusaria predominantly colonize plant material during the pre-harvest stage (Fig. 1a). Fungal colonies were observed on the straw collected from Herd 1 on Days 16 (Fig. 1b) and 72 (Fig. 1c) and showed typical characteristics of the *F. graminearum* species complex. However, typical colonies of *Aspergillus versicolor* or *Aspergillus nidulans* were not observed on any of the straw samples from Herd 1. Fungal contamination of straw collected from Herd 2 was very low; only one very small colony of *Penicillium* sp. was observed (Fig. 1d); these findings confirm the analytical results, as in the rice straw of this herd only very low amounts of STC could be detected that may results from previous, no longer traceable, fungal invasion.

DISCUSSION

Rice straw is the most important roughage used for beef cattle production in Japan, and STC is a major mycotoxin produced in rice. The harmful or chronic effects of STC in cattle are not well understood, and the toxin is not regulated or controlled in Japan. Our objectives were to provide preliminary data on the potential contamination of rice straw with STC and to assess the systemic exposure of cattle by analyzing urine samples.

A comparison of the measurable STC concentration in rice straw confirmed the contamination with STC, albeit at rather low levels, whereas no STC could be measure in the concentrate added to the diet. The analysis of urine samples from exposed animals indicated that STC is not degraded in the rumen and reaches the liver. This is in contrast to various other mycotoxins that are successfully inactivated by the rumen microorganisms, which protects the animal from exposure to various feed contaminations. This is in line with the clinical observations that ruminating cattle is less sensitive to many mycotoxins, including for example ochratoxin A and the group of trichothecenes. The most prominent example for another mycotoxin that is not degraded by the rumen flora is fumonisin B1 (Fink-Gremmels, 2008). The current study could not answer the question what fraction of the parent STC reaches the systemic circulation. Our preliminary studies clearly indicated that STC is extensively conjugated in the liver, presumably to glucuronic acid, as only trace amounts of the free mycotoxin could be detected in urine. This finding is of clinical relevance, as pre-systemic elimination of conjugates and their excretion either by bile, or with urine (as measured here), still effectively can reduce the amount of the toxin reaching the systemic circulation. The lack of significant adverse clinical symptoms in the exposed animals in this study seems to support this hypothesis.

There is a general consensus that most contaminated feed samples contain more than one mycotoxin (Monbaliu et al., 2010; van Pamel et al., 2011; Streit et al., 2012). Feed commodities might be contaminated with different types of fungi, each of which produce mycotoxins, and hence compound feeds contain many different products that contribute to the final mycotoxin profile (Fink-Gremmels, 2008; Streit et al., 2012). For example, Warth et al. (2012) recently reported that a commercial maize mill sample from Burkina Faso in Africa contained 6 μg/kg STC and 44 μg/kg ZEN, as well as 27 other mycotoxins. The samples investigated in this study had previously been shown to contain also the *Fusarium* toxin zearalenone and we observed that the chronic diarrhea in cattle of Herd 1 (MA1 group) improved between Days 58 and 72, a period during which the cattle received new rice straw, less contaminated with zearalenone (Hasunuma et al., 2012). Therefore, although many factors might have contributed to the improvement in the physical condition of the animals, the higher level of STC in rice straw from Herd 1, possibly in combination with ZEN contamination (>8 mg/kg), might be one possible reason for the continuous diarrhea in Herd 1 cattle. Vesonder and Horn (1985) previously reported acute clinical signs, including bloody diarrhea and death, in dairy cattle given STC-contaminated (8 mg/kg) feed infected with *A. versicolor*. The STC concentrations measured in our study were considerably lower, which might explain why no apparent severe clinical signs others than chronic diarrhea, which could also be attributed to other microbial infections, were noted in this study.

The growth of *Fusarium spp.* was correlated with ZEN concentrations in the straw sample from Herd 1, as we reported previously (Hasunuma et al., 2012). Interestingly, we did not observe the presence of *A. versicolor* or *A. nidulans*, both of which are known producers of STC, in the rice straw, even on Day 0. The only fungi present were *A. niger* and a small

colony of *Fusarium* spp. These results indicate that fungal infection patterns differ, even within the same lot of commercially available straw, and that the contamination patterns of each mycotoxin might largely depend on the type of dominant fungus in the feed. Rank et al. (2011) recently stated that STC is a key metabolite in mycotoxin research, and that new species that produce this toxin might be found. Therefore, although it is unclear why no fungal species known to produce STC were detected in the present study, more detailed studies that include different culture methods or molecular tools for fungal identification may be able to explain such apparent inconsistencies in the future.

Although we previously could report that the applied commercial mycotoxin adsorbent (mixed with the concentrate or given as topdressing) significantly reduced ZEN concentrations in the urine (Takagi et al., 2011; Hasunuma et al., 2012), no comparable protective effects of supplementary MA on STC absorption could be observed when measuring the STC contamination in urine as a marker. This result confirms the usefulness of urine analysis for the objective evaluation of the effects of supplemental MA on the bioavailability of mycotoxins. This finding also shows the efficacy of MA supplementation, as a measure to prevent mycotoxin absorption needs to be assessed for all individual mycotoxins, as significant differences can be expected.

In conclusion, to our knowledge, this is the first study showing that STC present in the diet of ruminating cattle is absorbed from the intestinal tract and passes the rumen. Results also show that the measurement of urinary concentrations of mycotoxins, including STC, is a reliable tool to monitor the exposure of cattle to contaminated feed. In addition, our results indicate that MA used in this study could not sequester STC. The observed lack of benefit from MA supplementation contrasts with our previous finding of the protective effect of MA

against ZEN adsorption. Considering the systemic bioavailability of STC in ruminating cattle, further field studies are required to improve our understanding about STC contamination and co-contamination with other mycotoxins, and to assess the potential risk for cattle health.

FIGURE LEGENDS

Figure 1. Czapek Dox agar culture showing typical colonies of *Aspergillus niger* in straw collected on Day 0 from Herd 1 (a). Czapek Dox agar culture showing typical colonies of *Fusarium graminearum* species complex in straw collected on Day 16 (b) and Day 72 (c) from Herd 1. Czapek Dox agar culture showing small colonies of *Penicillium spp.* in straw collected on Day 50 from Herd 2 (d).

Figure 2. Protocol for the supplementation of dietary feed with mycotoxin adsorbent (MA) and for the sampling of urine and feed samples. Sterigmatocystin concentrations in the urine and feed samples collected at each sampling point were analyzed using liquid chromatography-tandem mass spectrometry. Herd 1: Zearalenone (ZEN)-contaminated herd that had shown persistently higher mean ZEN concentrations in the urine. Herd 2: No ZEN contamination. Both herds were fed from the same lot of concentrate, but they received different rice straw. *MA1: MA was mixed with dietary concentrate. *MA2: MA was topdressed on the dietary concentrate. **MA1: Typical straw was replaced by a new lot of straw with less contamination. **MA2: Twice the recommended volume of MA was topdressed on the dietary concentrate. Control: No MA was provided during the experimental period.

Figure 3. Representative liquid chromatography-tandem mass spectrometry chromatograms for (a) the STC standard (10 ng/mL); (b) no detectable level of STC in the urine sample; and (c) a clear peak of STC contamination in the urine sample.

Figure 1

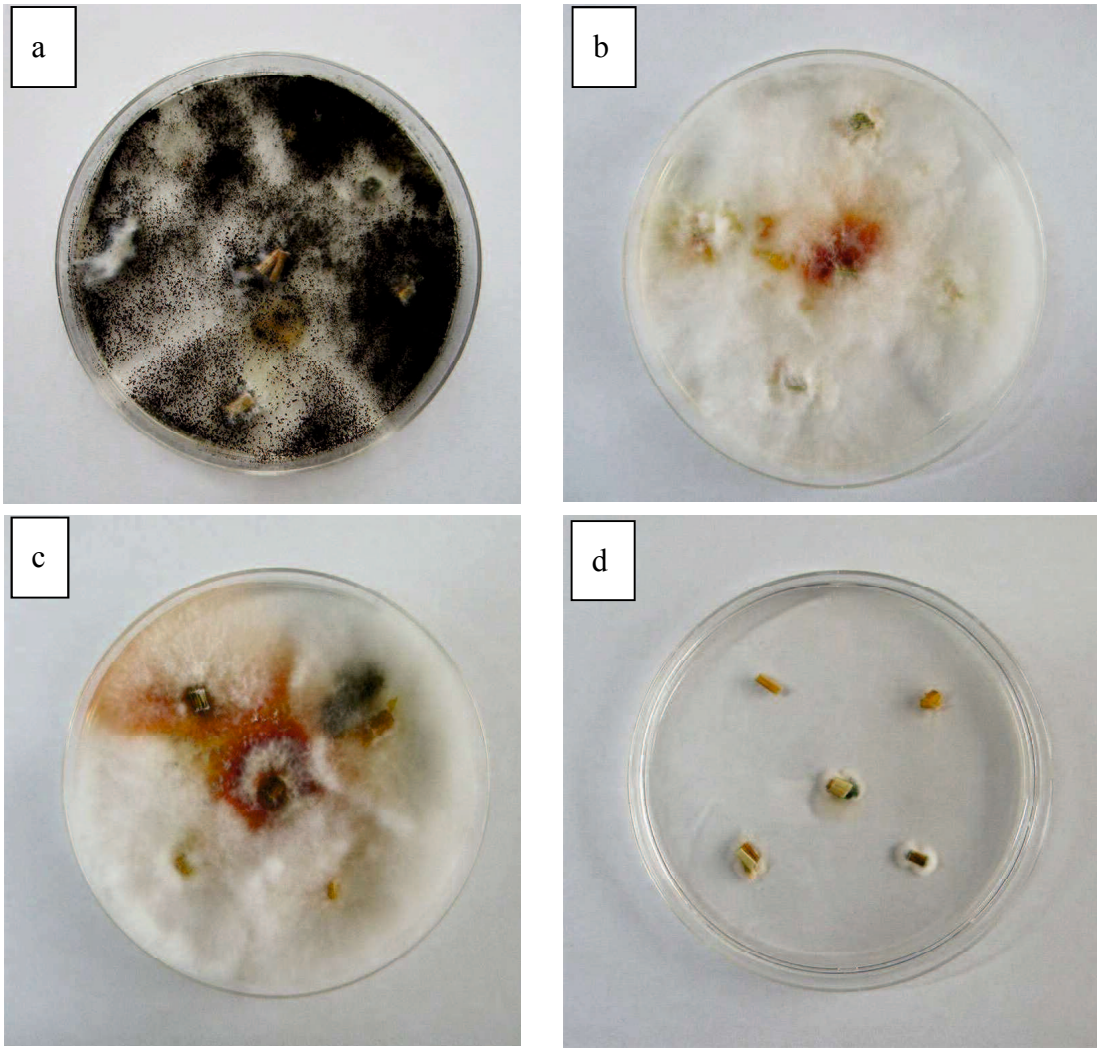

Figure 2

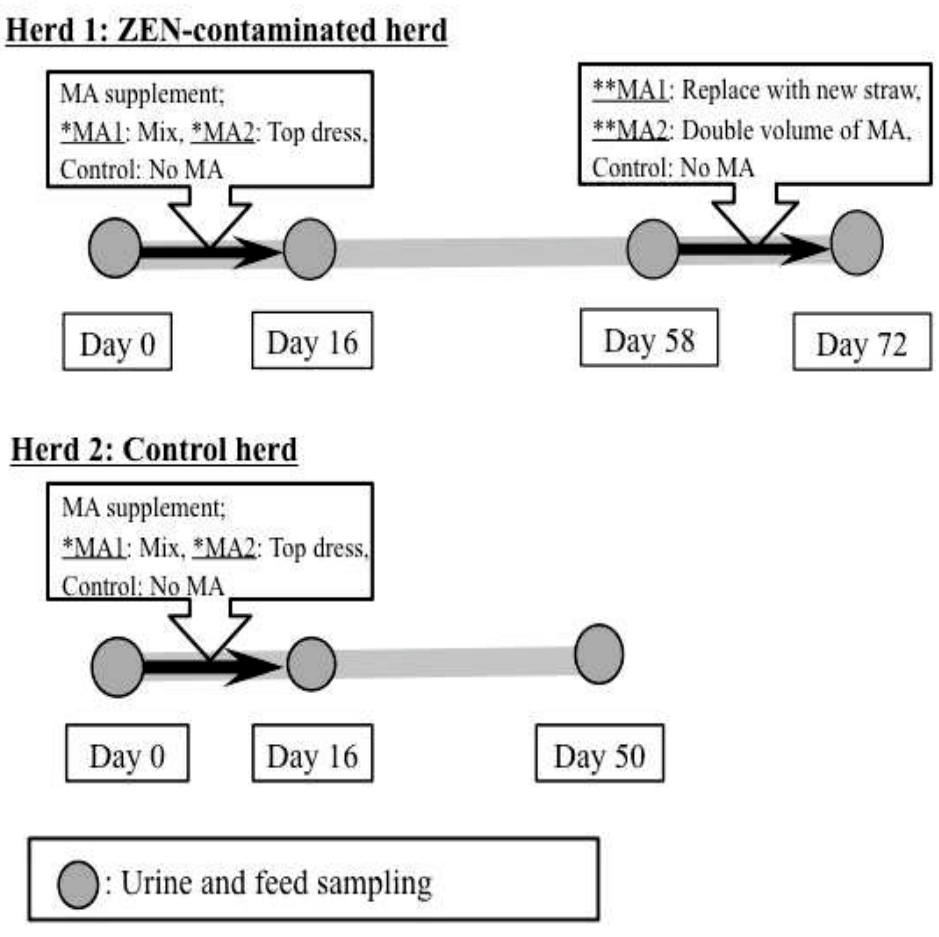
Herd 1: ZEN-contaminated herd
MA supplement;
*MA1: Mix, *MA2: Top dress,
Control: No MA
**MA1: Replace with new straw,
**MA2: Double volume of MA,
Control: No MA
Day 0
Day 16
Day 58
Day 72
Herd 2: Control herd
MA supplement;
*MA1: Mix, *MA2: Top dress,
Control: No MA
Day 0
Day 16
Day 50
: Urine and feed sampling

Figure 3

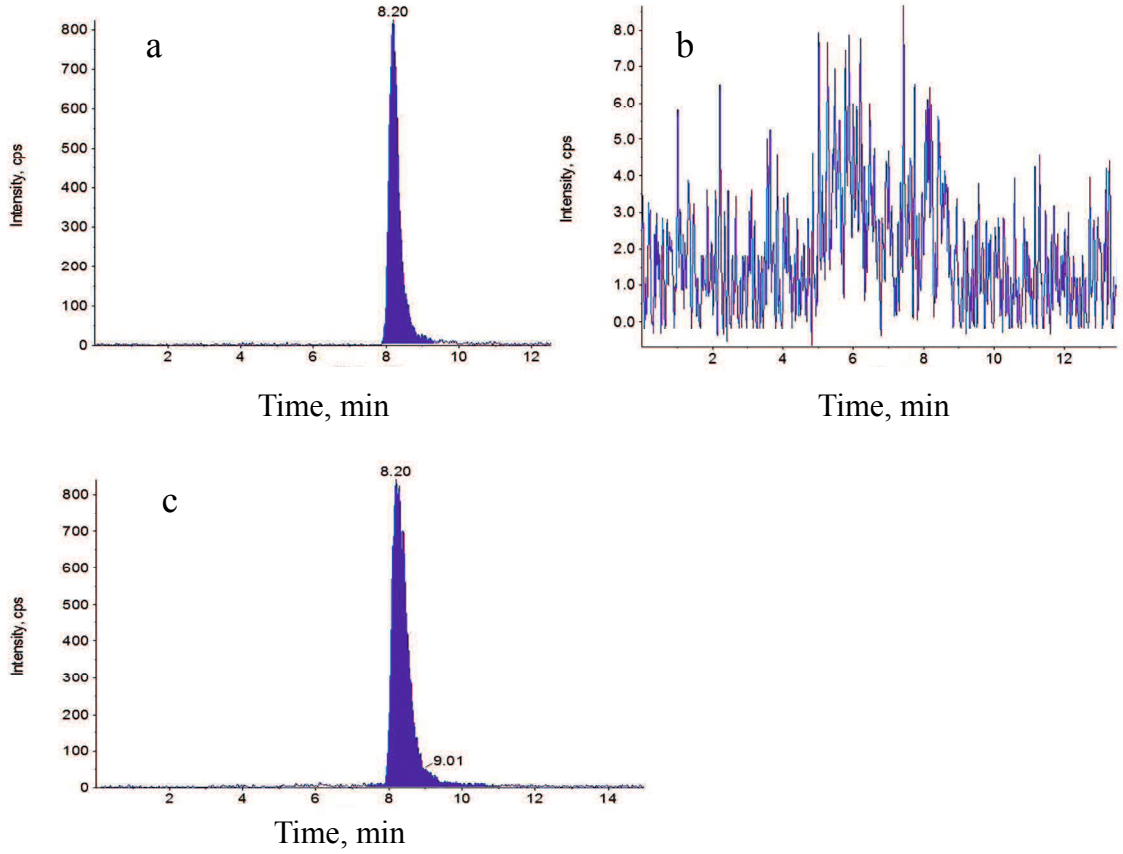
a
8.20
Intensity, cps
Time, min
b
Intensity, cps
Time, min
c
8.20
9.01
Intensity, cps
Time, min

Table 1. Results of the effects of the pre-incubation of urine samples with β-glucuronidase/arylsulfatase on the measurable urinary STC concentrations

	Urinary STC concentrations (pg/mg creatinine)	
	β-glucuronidase/arylsulfatase pre-incubation	
	With	Without
Urine sample		
Herd 1		
1	116.2	No peak
2	95.6	No peak
3	138.3	No peak
4	49.6	No peak
5	93.5	No peak
Mean (±SEM)	98.6 ± 14.7	–
Herd 2		
1	46.8	No peak
2	29.1	1.6
3	63.3	No peak
4	35.4	2.6
5	35.8	No peak
Mean	42.1 ± 6.0	2.1 ± 0.7

Table 2. Sterigmatocystin (STC) concentrations (measured as pg/mg creatinine) determined using liquid chromatography-tandem mass spectrometry in the urine of cattle and the effects of the application of a mycotoxin adsorbent (MA)

	Samples	**Day 0**	**Day 16**	**Day 58**	**Day 72**
Herd 1 (ZEN contamination)	STC in straw mg/kg	0.15	0.17	0.24	New: 0.04 Old: 0.21
	STC in concentrate mg/kg	<0.01	<0.01	<0.01	NT
	STC in urine				
	MA1	327 ± 26	209 ± 22	569 ± 111	33 ± 8[a]
	MA2	247 ± 57	239 ± 28	416 ± 62[c]	258 ± 33[b]
	Control	190 ± 43[a]	182 ± 43	898 ± 97[a,d]	354 ± 56[b]

	Samples	**Day 0**	**Day 16**	**Day 50**
Herd 2 (no ZEN contamination)	STC in straw mg/kg	0.06	0.03	<0.01
	STC in concentrate mg/kg	<0.01	<0.01	<0.01
	STC in urine			
	MA1	67 ± 22	147 ± 39	42 ± 19
	MA2	76 ± 22	86 ± 21	0
	Control	20 ± 13[b]	62 ± 23	0[b]

Within rows, different superscript letters (a-b, c-d) indicate significant differences ($P < 0.05$).

OVERALL DISCUSSION AND CONCLUSION

While mycotoxins in animal feeds may cause acute intoxications, their greatest impact is their negative effect on animal performance and productivity. Therefore, an objective assessment method using biological samples collected *in vivo* is necessary for monitoring the mycotoxin contamination/recovery in the cattle and is crucial for assessment of the dietary feeds and feeding management. We previously reported the practical usefulness of measuring urinary concentrations of ZEN and its metabolites not only for monitoring the natural ZEN contamination levels in cattle feed at the farm level, but also for *in vivo* assessment of MA function after supplementing feeds with MA (Takagi et al., 2011, Hasunuma et al., 2012). In this thesis, using practical application of our established urinary ZEN monitoring system for cattle herd was utilized to gain new insights regarding possible mycotoxin prevention methods at the farm levels, especially in beef cattle production.

The Chapter 1 describes the results of our field trial conducted within a cattle herd naturally contaminated with ZEA indicated that monitoring urinary concentrations of ZEA and its metabolites by LC/MS/MS is a useful tool for the objective evaluation of the effects of MA supplementation strategies in contaminated herds. The MA supplementation of dietary feeds, at least for inhibition of the effects of ZEA and its metabolites, preventive effects on ZEA absorption from the intestine of cattle may be affected by the method of supplementation, i.e. mixing with or topdressing with dietary feeds, even for naturally occurring ZEA contamination levels. It was also confirmed that chronic ZEA exposure in cattle might not be directly reflected by the results of haematological and serum biochemical analysis. The results

of this study suggest that MA supplementation has beneficial effects on recovery of liver function as reflected by AST and GGT levels. Additionally, by measuring AMH and P4 concentrations in cattle herds with and without naturally occurring ZEA contamination, it was hypothesized that ZEA (acting as an endocrine disrupter) may affect the population status of antral follicles in bovine ovaries without affecting the ovulation status of female cattle. Further studies involving a greater number of cattle herds are required to clarify the effect of ZEA on follicular development.

The study of Japanese Black breeding herds fed with ZEA-controlled feed management (below the permitted threshold levels) described in Chapter 2 suggests that a relationship exists between the contamination level of ZEA and its metabolites with the serum AMH concentration, even when ZEA dietary contamination is low. However, further studies are required, and these studies should include *in vivo* trials with greater numbers of cattle herds as well as *in vitro* co-exposure experiments in isolated bovine antral follicle cultures with both ZEA and its metabolites and other mycotoxins.

As described in Chapter 3, to our knowledge, this is the first study showing that STC present in the diet of ruminating cattle is absorbed from the intestinal tract and passes the rumen. Results also show that the measurement of urinary concentrations of mycotoxins, including STC, is a reliable tool to monitor the exposure of cattle to contaminated feed. In addition, our results indicate that MA used in this study could not sequester STC. The observed lack of benefit from MA supplementation contrasts with our previous finding of the protective effect of MA against ZEN adsorption. Considering the systemic bioavailability of

STC in ruminating cattle, further field studies are required to improve our understanding about STC contamination and co-contamination with other mycotoxins, and to assess the potential risk for cattle health.

In conclusion, the results of these field trials confirm that a system for monitoring urinary ZEN and STC concentrations is a useful tool for predicting the exposure of animals at the farm level to ZEN and STC. By applying the urinary monitoring system, it allows for the objective evaluation of MA supplementation to feeds at the farm level. Moreover, dietary contamination with mycotoxins may affect the population of antral follicle in the ovary. Further studies focusing on the collection of a large dataset are needed to enhance the understanding of the relationship between multi-mycotoxin contamination in the dietary feeds and reproductive efficacy of the cattle herds, and for the evaluation of suitable MAs for each cattle herd based on the urinary monitoring system.

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