On-farm Application of Urinary Mycotoxin Monitoring Systems for Feed Hygiene Management to Enhance Cattle Breeding Herd Productivity

飼料衛生管理による生産性向上を目的とした尿中マイコトキシン濃度測定系の繁殖牛群における適用

Joint Graduate School of Veterinary Medicine
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We hereby recommend that the thesis prepared under supervision by Oky Setyo Widodo, entitled "On-farm application of urinary mycotoxin monitoring systems for feed hygiene management to enhance cattle breeding herd productivity" should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFC	: Antral Follicle Count
AG	: Albumin/Globulin ratio
Alb	: Albumin
AMH	: Anti-Müllerian Hormone
APP	: Acute Phase Protein
BUN	: Blood Urea Nitrogen
Ca	: Calcium
CCR	: Cow Conception Rate
Cre	: Creatinine
DFA III	: Fructo-Oligosaccharide
DM	: Dry Matter
DNA	: Deoxyribonucleic acid
DOM-1	: Deepoxy-deoxynivalenol
DON	: Deoxynivalenol
DON-3G	: Deoxynivalenol-3-glucoside
DW	: Distilled Water
ELISA	: Enzyme-linked immunosorbent assay
ESI	: Electrospray ionization
et al.	: et alia, “and others”
FFA	: Free Fatty Acids
GGT	: Gamma-Glutamyl Transpeptidase
GLU	: Glucose
GOT	: Glutamate-Oxaloacetate Transaminase
HF	: Holstein Friesian
HPLC	: High-Performance Liquid Chromatography
IL-1	: Interleukin-1
IL-6	: Interleukin-6
IP	: Inorganic Phosphorus
IVF	: In Vitro Fertilization
JB	: Japanese Black cattle (Wagyu)
kDa	: Kilodalton
LC-MS/MS	: Liquid Chromatography with tandem mass spectrometry
LoD	: Limit of Detection
MAs	: Mycotoxin Adsorbents
Mg	: Magnesium
mL	: Milliliter
mM	: Millimolar
MOET	: Multiple Ovulation and Embryo Transfer
N/A	: Not available
ND	: Not Detected

NIV	: Nivalenol
nm	: Nanometers
OPU-IVP	: Ovum Pick Up-In Vitro Embryo Production
P4	: Hormone Progesterone
PBMC	: Peripheral Blood Mononuclear Cells
pg/mL	: Picogram/milliliter
pH	: Potential of Hydrogen, “acidity”
ppt	: Part per trillion
rpm	: revolutions per minute
SAA	: Serum Amyloid A
SCC	: Somatic Cell Count
SEM	: Standard Error of the Mean
SPE	: Solid-Phase Extraction
STC	: Sterigmatocystin
T-Cho	: Total Cholesterol
TG	: Triglycerides
TMR	: Total Mixed Rations
TNF-α	: Tumor Necrosis Factor-α
TP	: Total Protein
VAR	: Vector autoregression
Vit.A	: Vitamin A
Vit.E	: Vitamin E
WCS	: Whole Crop Silage
ZEN	: Zearalenone
°C	: Degree Celsius
15-Ac-DON	: 15-acetyldeoxynivalenol
3-Ac-DON	: 3-acetyldeoxynivalenol
3HB	: 3-hydroxybutyrate
8-OHdG	: 8-hydroxy-2′-deoxyguanosine
α-ZEL	: α-zearalenol
β-ZEL	: β-zearalenol
µL	: Microliter

ABSTRACT

In the first series of experiments, we address an advantageous application of a urinary zearalenone (ZEN) monitoring system not only for surveillance of ZEN exposure at the production site of breeding cows but also for follow-up monitoring after improvement of feeds provided to the herd. As biomarkers of effect, serum levels of the anti-Müllerian hormone (AMH) and serum amyloid A (SAA) concentrations were used. Based on the results of urinary ZEN measurement, two cows from one herd had urinary ZEN concentrations which were two orders of magnitude higher (ZEN: 1.34 mg/kg, sterigmatocystin (STC): 0.08 mg/kg in roughages) than the levels of all cows from three other herds (ZEN: not detected, STC: not detected in roughages). For the follow-up monitoring of the herd with positive ZEN and STC exposure, urine, blood, and roughage samples were collected from five cows monthly for one year. A monitoring series in the breeding cattle herd indicated that feed concentrations were not necessarily reflected in urinary concentrations; urinary monitoring assay by ELISA may be a simple and accurate method that reflects the exposure/absorption of ZEN. Additionally, although the ZEN exposure level appeared not to be critical compared with the Japanese ZEN limitation in dietary feeds, a negative regression trend between the ZEN and AMH concentrations was observed, indicating that only at extremely universal mycotoxin exposure levels, ZEN exposure may affect the number of antral follicles in cattle. A negative regression trend between the ZEN and SAA concentrations could also be demonstrated, possibly indicating the innate immune suppression caused by low-level chronic ZEN exposure. Finally, significant differences ($p = 0.0487$) in calving intervals between pre-ZEN monitoring (mean ± SEM: 439.0 ± 41.2) and post-ZEN monitoring (349.9 ± 6.9) periods were observed in the monitored five cows. These preliminary results indicate that the urinary ZEN monitoring system may be a useful practical tool not only for detecting contaminated herds under field conditions but also provides an initial look at the effects of long-term chronic ZEN/STC (or other co-existing mycotoxins) exposure on herd productivity and fertility.

The second series of experiments, a herd of Japanese Black (JB) breeding cattle with sporadic reproductive disorders was continuously monitored for an additional year to assess the effects of the urinary ZEN concentration and changes in parameters (AMH and SAA) with time-lag variables and herd fertility (reproductive performance). This herd had high (exceeded the Japanese dietary feed regulations) urinary ZEN and rice straw ZEN

concentrations (1.34 mg/kg). Long-term data of the herd with positive ZEN exposure revealed a decreasing ZEN concentration in urine and a gradual decrease in the AMH level with age. The AMH level was significantly affected by the ZEN value 2-months earlier and the AMH level in the previous month. The changes in ZEN and SAA values were significantly affected by the ZEN and SAA values in the previous month. Additionally, calving interval data between pre-monitoring and post-monitoring showed a significantly different pattern. Furthermore, the calving interval became significantly shorter between the time of contamination (2019) and the end of the monitoring period (2022). In conclusion, the urinary ZEN monitoring system may be a valuable practical tool for screening and detecting herd contamination in the field, and acute and/or chronic ZEN contamination in dietary feeds may affect herd productivity and the fertility of breeding cows.

The third series of experiments, widespread presence of *Fusarium* mycotoxins in animal feed is a global issue, not only for the health of livestock but also for ensure the safety of food as an end product. High concentrations of ZEN and deoxynivalenol (DON) have been detected in the diets of JB and Holstein Friesian (HF) breeding herds. Consequently, we monitored serum biochemical parameters over a long time in both herds, focusing on AMH levels and acute-phase inflammation. Additionally, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and progesterone levels were measured in the HF herd. The JB herd, a ZEN-dominant model with low DON contamination, demonstrated ZEN levels that exceeded the Japanese limit in the purchased total mixed rations (TMR). Conversely, the HF herd, which primary consumes DON-dominant feed with low ZEN contamination, had high DON levels in the dent corn silage. Specifically, the JB herd's TMR contained 1.79 mg/kg ZEN and 0.58 mg/kg DON, whereas the HF herd's silage had 15.3 mg/kg DON (dried sample) and 0.1 mg/kg ZEN. Enzyme-linked immunoassay was used to measure urinary ZEN-DON levels following confirmation through liquid chromatography-tandem mass spectrometry. Urinary ZEN-DON levels measured were significantly correlated ($p < 0.05$, $r > 0.6$) in both herds. In the HF herd, AMH levels increased ($p = 0.01$) and serum amyloid A (SAA) levels decreased ($p = 0.02$) when contaminated and at the end of the monitoring period. Additionally, urinary ZEN and DON levels were significantly correlated with SAA levels (ZEN: $p = 0.00$, $r = 0.46$; DON: $p = 0.03$, $r = 0.33$), with an increase in ZEN and DON levels resulting in higher SAA levels. The JB herd showed no significant differences. Additionally, in the HF herd, 8-OHdG/Cre levels increased significantly during major contamination periods ($p < 0.05$). Clinical data from the HF herd indicated an increase in mastitis cases and treatment rates during periods of major contamination. Abortion rates in

the HF herd decreased from 22.9% (before monitoring) to 8.9% (during the high contamination period) and finally to 1% (at the end of the monitoring period), with corresponding increases in progesterone levels. ZEN-DON contamination adversely affects breeding cattle's productivity, reproductive performance, and health. Therefore, monitoring urinary ZEN-DON is valuable for detecting contaminants and ensuring the safety of food products.

In conclusion, ZEN-DON contamination can adversely affect the productivity, reproductive efficacy, and health of breeding cattle. We examined the correlation of contamination measurements by ELISA and LC-MS/MS methods. The parallel urinary ZEN and DON contamination monitoring system we developed is effective for screening and detecting acute and/or chronic contamination of cow breeding herds on farms, hence ensuring the safety of food products.

GENERAL INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi. The widespread presence of *Fusarium* mycotoxins in animal feed is a global concern for livestock health and reproduction, as well as the safety of food in the final product. Global warming and/or climate change are discussed as potential reasons for higher exposure rates, which may enhance the risk of harmful effects on both human and animal health [Liew *et al.,* 2018; Vandicke *et al.,* 2019; Li *et al.,* 2020; Raduly *et al.,* 2020]. Mycotoxin's contamination occurs at several stages, including field growth, harvest, transportation, feed production, and storage [Alonso *et al.,* 2013; Liu *et al.,* 2020; Vandicke *et al.,* 2021]. *Fusarium* species produce two primary mycotoxins: zearalenone (ZEN) and deoxynivalenol (DON). ZEN is an estrogenic mycotoxin that interferes with cattle reproductive functions and is frequently found in grains and feed [Gruber-Dorninger *et al.,* 2019; Eskola *et al.,* 2019; Thapa *et al.,* 2021]. Although fungal growth and ZEN synthesis may continue during poor storage conditions, is primarily formed during the pre-harvest stage [Liu *et al.,* 2020]. DON is cytotoxic and immunotoxic and can impair protein synthesis. Susceptibility to DON varies by animal, with ruminants being rather resistant and pigs being particularly vulnerable [Pestka, 2007]. *Fusarium*-derived mycotoxins, specifically zearalenone (ZEN) and deoxynivalenol (DON), as well as their co-occurrence (ZEN-DON), should be closely monitored [Gruber-Dorninger *et al.,* 2019].

Managing cows to prevent mycotoxin contamination is essential to reducing its adverse effects on reproductive performance. Since regulating animals' exposure to mycotoxins under farm conditions, where feed supplies can vary quickly, urinary monitoring concentrations of ZEN and DON is considered an appropriate biomarker. Previously, we have established a urinary ZEN-monitoring system with enzyme-linked immunoassay (ELISA) for initial screening purposes, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) validation to detect ZEN and its related metabolites [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012]. Additionally, we have reported that monitoring ZEN or sterigmatocystin (STC) levels in urine is not only a practical and useful way of evaluating and detecting the naturally contamination status of cattle herds, but also assessing the efficiency of mycotoxin adsorbents (MAs) supplemented in dietary feed to reduce intestinal absorption of mycotoxins [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012; Toda *et al.,* 2018; Sasazaki *et al.,* 2021]. We have continued to monitor cattle herds on the farm, using the

urinary monitoring system to detect subclinical contaminated herds. During this monitoring, we detected three herds that consumed roughage contaminated with high levels of ZEN and/or DON, surpassing the standard levels in Japan. The feed contamination limit for ZEN is 1 mg/kg, whereas the limit for DON is 4 mg/kg for calves older than 3 months. We conducted long-term (25 months) monitoring of one group of JB cows contaminated with ZEN (single contamination) in Chapters 1 and 2, and monitored both groups of Japanese Black (JB) and Holstein Friesian (HF) cows contaminated with ZEN and DON (co-contamination) in Chapter 3. In Chapter 3, we use these two groups of cattle (JB and HF) as models for ZEN- and DON-dominant contamination.

Our study investigated the impact of ZEN and/or DON contamination on various biomarkers: anti-Müllerian hormone (AMH), a potential marker of fertility, ovulatory hyper-response, ovarian dysfunction, and herd longevity [Mosa *et al.,* 2017; Umer *et al.,* 2019; Ramesha *et al.,* 2022]; serum amyloid A (SAA), an acute-phase protein associated with inflammation [Eckersall *et al.,* 2010; Chan *et al.,* 2010; Bazzano *et al.,* 2022; Shinya *et al.,* 2022]; blood biochemical tests; measuring levels of progesterone (P4); and urinary 8-hydroxy-2'- deoxyguanosine (8-OHdG), a biomarker of DNA oxidative stress [Giromini *et al.,* 2016; Olarotimi et al., 2023]. Furthermore, we verified the relationship between changes in calving interval, conception rate (CCR), abortion rate, and disease incidence based on the clinical records of herds.

AMH is a 140 kDa glycoprotein belonging to the transforming growth factor-beta superfamily. It is secreted by the ovarian granulosa cells primarily from the preantral and early antral follicles [Monniaux *et al*., 2008, 2012]. AMH is an endocrine marker that is closely associated with gonadotropin-responsive ovarian reserves and pool size of the growing preantral and small antral follicles [Monniaux *et al.,* 2012]. Additionally, recent studies indicate that blood AMH levels vary among individual cows, and that they are reliable endocrine markers for the number of ovulation events and embryos produced in both multiple ovulation and embryo transfer (MOET) and/or ovum pick up-in vitro embryo production (OPU-IVP) programs [Monniaux *et al.,* 2010; Ireland *et al.,* 2011; Fushimi *et al.,* 2019; Mossa *et al.,* 2019].

An acute phase protein (APP) is a specific protein whose blood concentration fluctuates within a short period when the body is subjected to harmful stimuli, such as infection or tissue damage. Previous reports indicated that APP are not only useful for monitoring inflammatory processes for diagnostic and prognostic purposes but also for analyzing various non-inflammatory conditions, such as pregnancy, parturition, metabolic

diseases, and both environmental and management stress [Murata *et al.,* 2004; Huzzey *et al.,* 2015]. SAA, similar to any other APP, such as C-reactive protein and haptoglobin, is now clinically used as an inflammatory marker in dairy and beef cattle practice [Alsemgeest *et al.,* 1994; Horadagoda *et al.,* 1999; Petersen *et al.,* 2004; Ceciliani *et al.,* 2012; Abdallah *et al.,* 2016]; moreover, SAA reportedly responded most rapidly to infection. Therefore, SAA can be used as a potential marker in distinguishing the severity of inflammation [Heegaard *et al.,* 2000].

Therefore, Chapter 1 (first-year results) and Chapter 2 (first-year results and second-year results, long-term monitoring for 25 months) were conducted to (1) re-evaluate and elucidate the urinary ZEN monitoring system for its practical usefulness on farm conditions and (2) evaluate the long-term monitoring results, concomitant with the relationship between changes in both naturally occurring urinary mycotoxins, particularly ZEN, and serum AMH concentration. Finally. in Chapter 3, we confirmed the effectiveness of the DON measurement system in urine samples using a commercially available ELISA kit. Furthermore, we performed blood biochemical analyses, assessing P4 levels and urinary 8-OHdG in a contaminated herd condition. We also examined the relationship between changes in the cow conception rate (CCR), abortion rates, disease incidence, and mycotoxin levels in urine and feed, based on clinical data from both herds (JB and HF).

Chapter 1

Practical application of urinary zearalenone monitoring system for feed hygiene management of a Japanese black cattle breeding herd— the relationship between monthly anti-Müllerian hormone and serum amyloid A concentrations

ABSTRACT

This study addresses an advantageous application of a urinary zearalenone (ZEN) monitoring system not only for surveillance of ZEN exposure at the production site of breeding cows but also for follow-up monitoring after improvement of feeds provided to the herd. As biomarkers of effect, serum levels of the anti-Müllerian hormone (AMH) and serum amyloid A (SAA) concentrations were used. Based on the results of urinary ZEN measurement, two cows from one herd had urinary ZEN concentrations which were two orders of magnitude higher (ZEN: 1.34 mg/kg, sterigmatocystin (STC): 0.08 mg/kg in roughages) than the levels of all cows from three other herds (ZEN: not detected, STC: not detected in roughages). For the follow-up monitoring of the herd with positive ZEN and STC exposure, urine, blood, and roughage samples were collected from five cows monthly for one year. A monitoring series in the breeding cattle herd indicated that feed concentrations were not necessarily reflected in urinary concentrations; urinary monitoring assay by enzyme-linked immunosorbent assay (ELISA) may be a simple and accurate method that reflects the exposure/absorption of ZEN. Additionally, although the ZEN exposure level appeared not to be critical compared with the Japanese ZEN limitation in dietary feeds, a negative regression trend between the ZEN and AMH concentrations was observed, indicating that only at extremely universal mycotoxin exposure levels, ZEN exposure may affect the number of antral follicles in cattle. A negative regression trend between the ZEN and SAA concentrations could also be demonstrated, possibly indicating the innate immune suppression caused by low-level chronic ZEN exposure. Finally, significant differences ($p = 0.0487$) in calving intervals between pre-ZEN monitoring (mean ± SEM: 439.0 ± 41.2) and post-ZEN monitoring (349.9 ± 6.9) periods were observed in the monitored five cows. These preliminary results indicate that the urinary ZEN monitoring system may be a useful practical tool not only for detecting contaminated herds under field conditions but also provides an initial look at the effects of long-term chronic ZEN/STC (or other co-existing mycotoxins) exposure on herd productivity and fertility.

INTRODUCTION

Recently, increasing attention has been paid to the impact of *Fusarium*-derived mycotoxins, as their prevalence seems to increase worldwide, despite efforts to minimize their concentration in animal feeds. Global warming and/or climate change are discussed as possible causes for higher exposure rates which may enhance the risk of harmful effects on both human and animal health [Liew *et al*., 2018; Vandicke *et al.,* 2019; Li *et al.,* 2020; Raduly *et al.,* 2020]. Indeed, a large-scale global survey of mycotoxin contamination in more than 70,000 sample feeds collected from more than one hundred countries suggested that mycotoxins are almost ubiquitously detected contaminants [Gruber-Dorninger *et al.,* 2019]. Gruber-Dorninger *et al.,* (2019) concluded that co-occurrence of *Fusarium*-derived mycotoxins (such as zearalenone (ZEN) and deoxynivalenol (DON) as the most important combinations) should be monitored more closely. Based on a recent review, ZEN, one of the *Fusarium*-derived estrogenic-mycotoxins, is mainly formed at the pre-harvest stage, although continued fungal growth and ZEN synthesis may continue during poor storage conditions [Liu *et al.,* 2020]. As controlling animals' exposure to mycotoxins is often difficult under farm conditions, where feed supplies may change rapidly, monitoring of urinary concentration of ZEN of farm animals such as cattle is presumed to be a suitable biomarker for ZEN exposure [Prelusky *et al.,* 1989; Usleber *et al.,* 1992; Kleinova *et al.,* 2002]. Previously, we have established a urinary ZEN-monitoring system with ELISA for initial screening purposes, followed by LC-MS/MS validation to detect ZEN and its related metabolites; α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) as well as sterigmatocystin (STC), as both toxins might occur together in diets for Japanese cattle [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012; Fushimi *et al.,* 2014]. Additionally, we have reported that monitoring ZEN or STC levels in urine is not only a practical and useful way of evaluating and detecting the naturally contamination status of cattle herds, but also assessing the efficiency of mycotoxin adsorbents (MAs) supplemented in dietary feed to reduce intestinal absorption of mycotoxins [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012; Toda *et al.,* 2018; Sasazaki *et al.,* 2021].

Multiple factors influence fungal growth and mycotoxin formation, including season, geographical location, drought, harvest time, processing, storage, and distribution, etc., [Liu *et al.,* 2020], thus, the important first step in combating mycotoxins, especially in herds fed homegrown forage begins with measuring the level/status of mycotoxins contamination in the feed of individual herds during the stage of sub-clinical health condition. We have

continued to monitor cattle herds in the field to detect subclinical ZEN-contaminated herds by using urinary ZEN monitoring. During this monitoring, we identified one herd which was speculated to have been fed rather high ZEN-contaminated roughage exceeding the standard value in Japan (>1 mg/kg) with urine samples by ELISA, following validated by LC-MS/MS assay of the dietary roughage. Given ethical and animal welfare concerns, and the high costs involved, it is hardly possible to conduct feeding trials with cattle exposed to ZEN contaminated feed to investigate the effects of chronic low levels of ZEN contamination. Therefore, we evaluated whether the identified cattle herd may serve as a useful tool for observing the effect of long-term exposure on urinary excretion of toxins as well as an indicator of the reproductive performance of female cattle.

The objectives of this field study were to (1) re-evaluate the urinary ZEN monitoring system for its practical usefulness in cattle farm conditions and (2) evaluate the follow-up monitoring results for 1 year, concomitant with the relationship between changes in both naturally occurring urinary ZEN and serum anti-Müllerian hormone (AMH) concentration, and STC concentrations. AMH is secreted by ovarian granulosa cells primarily from preantral and early antral follicles of females and is an endocrine marker closely associated with both gonadotrophin-responsive ovarian reserves and with the size of the pool of growing preantral and small antral follicles [Monniaux *et al.,* 2008, 2013]. Additionally, serum amyloid A (SAA), which is one of the most reliable acute phase proteins (APPs) primarily produced by the liver induced by the inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α [Berg *et al.,* 2011; Zhang *et al.,* 2018], was measured to monitor inflammation in each cow at the monthly sampling time, given our previous report that not only calving itself but also severe inflammation during the postpartum period indicated by high SAA concentration can affect the AMH concentration in cows [Okawa *et al.,* 2021].

MATERIAL AND METHODS

All experiments were conducted according to the guidelines and regulations for the protection of experimental animals and guidelines stipulated by Yamaguchi University, Japan (no. 40, 1995; approved on 27 March 2017) and informed consent was obtained from the farmers.

Chemicals and Solvents

ZEN was purchased from MP Biomedicals (Heidelberg, Germany). The metabolites α-ZEL and β-ZEL were purchased from Sigma (St. Louis, MO, USA). Stock solutions of ZEN, α-ZEL, and β-ZEL, each at a concentration of 1 μg/mL in methanol, were stored under light protection at 4 ˚C. 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, and high-performance liquid chromatography (HPLC)-grade methanol was purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). β-Glucuronidase/arylsulfatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan), and Tris was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Screening by Urinary ZEN Monitoring to Detect Cattle Herds Fed with Dietary Roughage with Elevated ZEN Contamination

Before the rice harvest period in September 2019, this screening was conducted at the Japanese Black (JB) breeding cattle production site to monitor the extent of ZEN contamination of rice straw and/or whole crop silage (WCS) stored by cattle farmers in the summer season when the mean temperature of daytime is higher than 30 ˚C. At the request of the managing veterinarian, urinary ZEN monitoring was performed in four herds (A, B, C, and D) of JB cows kept for breeding in the neighborhood in the Kyushu area, Japan, for which the veterinarian routinely provides veterinary treatments and consults with four farmers. All animals were housed indoors, and roughage and concentrates were fed separately. Feeding and management systems were similar in each herd and the dates of sampling and contents of the feeds in each herd are detailed in **Table 1**. As feed intake may reflect the ZEN exposure, urine samples were collected from two cows with similar body weight within each herd during natural urination after softly massaging the perineum. Regarding the number of cows to be sampled for urine in each herd, referring to our previous

report [Takagi *et al.,* 2011], we considered samples from two cows to be sufficient to evaluate and estimate the contamination status of feed fed the same amount and same lot of feed. In addition, samples of all roughages, such as rice straw and WCS, were obtained from each herd to measure both ZEN and STC concentrations in the roughage. All concentrates fed to cattle in each herd were purchased from feed companies and are generally tested for mycotoxin contamination during the manufacturing stage. The urine and roughage samples were immediately placed into a cooler, protected from light, transported to the clinic office, and frozen. The frozen samples were sent to our laboratory and stored at −30 ˚C until our analysis of ZEN and creatinine (Cre) concentrations in the urine, and ZEN and STC concentrations in the roughage.

Table 1. Composition of feeds provided to the monitored herds kept for breeding purposes.

Herd	Date of Sample Collection	Forage Feeds/Day	Formula Feeds/Day
A (n = 2) (Both 12 y)*	10 July 2019	Home-grown rice straw 2 kg, Home-grown WCS (rice) 6 kg, Home-grown Italian ryegrass 4 kg Total: 12 kg	Commercially available concentrates 4 kg
B (n = 2) (3 y and 5 y)	24 June 2019	Home-grown rice straw 10 kg, Mixed of Italian ryegrass and Orchard grass 10 kg Total: 20 kg	Commercially available concentrates 1 kg, Wheat bran 1 kg, Maize 1 kg
C (n = 2) (8 y and 10 y)	19 August 2019	Home-grown rice straw 12~14 kg, Orchard grass 10 kg (once a week) Total: 12~14 kg	Commercially available concentrates 3 kg Wheat 0.5–1 kg
D (n = 2) (9 m and 10 m)	11 July 2019	Imported Oats-hey 2.25 kg, Bermuda-grass 2.25 kg Total: 4.5 kg	Commercially available concentrates 4.5 kg

*Age of the breeding cattle at sampling, y; years old, m; month old. WCS: whole crop silage.

Follow-up Monthly Monitoring on the Breeding Cattle Herd with Known Feed Contamination

Since contamination of rice straw/WCS from herd C collected in August 2019 exceeded the standard value of ZEN ≥ 1 mg/kg concomitant with STC was detected, this herd was selected for further monitoring. Therefore, monthly regular urinary ZEN monitoring of herd C was performed from July 2020 to help determine whether similar ZEN and STC exposure from the rice straw/WCS occurred year to year. For monitoring, five cows (Cows 1 to 5: mean 5.0 y: 3.6–6.3 y) in Herd C with similar body weight (approximately

500 kg) fed with the same roughage and concentrated feed were selected and monthly urine, blood, and roughage were sampled. We collected both urine and blood samples from the five cows at the beginning of each month, approximately 2 h after the morning feed, as per our previous methodology [Takagi *et al.,* 2011], and we also collected roughage samples fed to these cows. Both urine and blood samples were immediately stored on ice, protected from light, and transported to the laboratory, and were stored at –30 ˚C after centrifugation as dispensed urine and serum in microtubes until analysis. The collected roughages were also stored at –30 ˚C until measurement of both ZEN and STC concentrations.

Zearalenone concentrations in the collected urine samples were measured by ELISA every two months, as described below, and urine samples were measured monthly when deemed necessary by the herd manager monitoring the condition of the roughage being fed or by contamination status of the roughage by fungi at the monthly sampling. During the follow-up period, daily feeding was performed while sharing the urinary ZEN concentration measurement results with the herd manager and the managing veterinarian. When a high urinary ZEN concentration was confirmed, the roughage lot fed at the time of sampling was changed, and the urinary ZEN concentration was measured again in the following month for follow-up purposes, concomitant with measurement of both ZEN and STC concentrations of roughage samples by LC-MS/MS as mentioned below. Concentrations of urinary ZEN, its metabolites, α-ZEL, β-ZEL, and STC of all collected urine samples during the follow-up period were measured by LC-MS/MS within one assay for reconfirmation of results by the ELISA assay and urinary STC measurement. A schematic representation of the experimental design is sown in **Figure 1**.

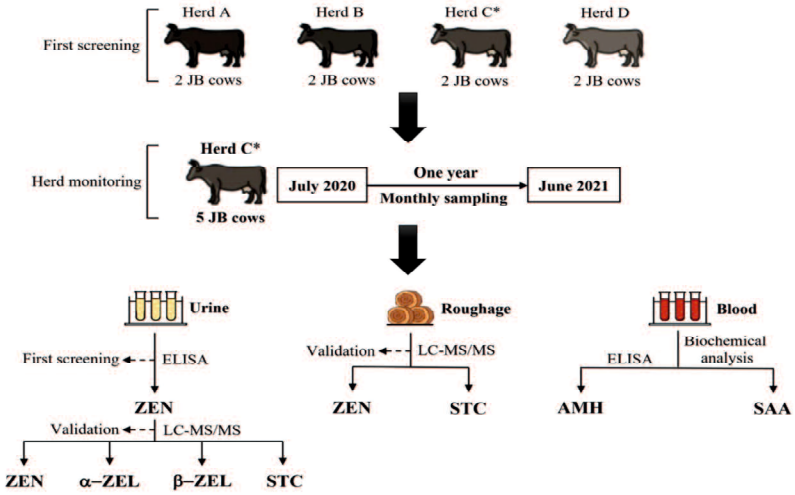

Figure 1. Schematic representation of the experimental design. C*, herd with high contamination: urinary ZEN concentrations exceeding the standard concentration of the ELISA kit, and ZEN-

contaminated roughage exceeding the standard value in Japan (>1 mg/kg). α-ZEL: α-zearalenol; β-ZEL: β-zearalenol; AMH: anti-Müllerian hormone; SAA: serum amyloid A; STC: sterigmatocystin; ZEN: zearalenone.

Reproductive Records

As a reproductive record, the calving intervals of the herd were compared for each year from 2017 to 2021. Additionally, the reproductive records from the five cows examined between pre-ZEN monitoring (2017 to 2019) and post-ZEN monitoring periods (2020 and 2021) were evaluated to confirm the impact of introducing the ZEN monitoring system on herd fertility.

Analytical Methods of ZEN in Urine and Feed Samples

Zearalenone concentration in urine was determined using a commercially available kit (RIDASCREEN Zearalenon; R-Biopharm AG, Garmstadt, Germany) according to the manufacturer's instructions, with minor modifications. Briefly, a urine sample (0.1 mL: 5-fold dilution of the kit) was added into 3 mL of 50 mM sodium acetate buffer (pH 4.8) and the solution was incubated for 15 h at 37 ˚C in the presence of 10 µL of β-glucuronidase/arylsulfatase solution. Thereafter, the samples were loaded onto a C18 solid-phase extraction (SPE) column (Strata; Phenomenex, Torrance, CA, USA), which had been preconditioned with 3 mL of methanol, followed by 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20). After washing the SPE column with 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20) and 3 mL of methanol (40%), the column was centrifuged for 10 min at 500 × *g* to dry the column. The analytes were then eluted slowly (flow rate: 15 drops/min) with 1 mL of methanol (80%). The eluate was evaporated to dryness at 60 ˚C using a centrifugation evaporator. The dried residue was redissolved in 50 µL of methanol, 450 µL of sample dilution buffer was added, the solution was mixed thoroughly, and an aliquot of 50 µL was used for the ELISA assay. To determine the ZEN concentration in the urine sample, RIDA SOFT Win (R-Biopharm) was used to calculate the absorbance at 450 nm using a microplate spectrophotometer. The cross-reactivity rates using this particular ELISA kit for α-ZEL, β-ZEL, and Zeranol were 41.6%, 13.8%, and 27.7%, respectively, based on the manufacturer's instruction, and the mean recovery rate of the ELISA assay based on the three trials was 84 ± 14%.

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 using a 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan). All urine

concentrations were expressed as a ratio of creatinine (pg/mg creatinine), as described previously [Takagi *et al.,* 2011].

Based on the results of the first screening and measurement of urinary ZEN concentrations by ELISA, both the urine and roughage samples in herds expected to have high ZEN infiltration in the feed were retested using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement system not only for the confirmation of the ELISA results but also for measuring the ZEN metabolites, α-ZEL and β-ZEL. Additionally, as per our previous reports, urinary STC levels were concomitantly higher in cattle fed ZEN-contaminated rice straw in Japan; thus, it was speculated that co-contamination of both ZEN and STC was observed. Therefore, in the retest, the STC concentration in urine and roughage was also measured according to our previous reports [Takagi *et al.,* 2011; Fushimi *et al.,* 2014].

The LC-MS/MS method and validation have been described in our previous report [Takagi *et al.,* 2011]. Briefly, each urine sample (0.5 mL) was mixed with 3.0 mL of 50 mM ammonium acetate buffer (pH 4.8) and 8 μL of glucuronidase/arylsulfatase solution and incubated for 12 h at 37 ˚C. The solution was loaded onto a C18 SPE column, which was preconditioned with 3 mL 100% methanol and 2 mL Tris buffer, followed by the addition of 2 mL Tris buffer and 3 mL of 40% methanol. After washing the SPE column with approximately 1 mL of 80% methanol, the volume of the eluted solution was adjusted to 1 mL. Then, 20 μL of the reconstituted solution was injected into the LC-MS/MS system. The LC-MS/MS analyses were performed on an API 2000 MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) interface and a 1200 Infinity Series HPLC system (Agilent Technologies, Santa Clara, CA, USA). The detection limits for ZEN, α-ZEL, and β-ZEL were 0.04 ng/mL, 0.05 ng/mL, and 0.05 ng/mL, respectively, while the mean recovery rates for ZEN, α-ZEL, and β-ZEL were 90%, 109%, and 90%, respectively. STC concentrations of the same eluted solution described above were also determined by LC-MS/MS using an API 2000 system equipped with an ESI as previously described [Fushimi *et al.,* 2014]. Briefly, after elution with approximately 1 mL of 80% methanol, the volume was adjusted to exactly 1 mL, and 20 μL of the 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. A mobile phase consisting of methanol/water/acetic acid (97:3:0.01, v:v:v) was used (200 μL/min) to separate the analyte in isocratic mode. Measurements were performed for 15 min.

The limit of detection (LoD) was 0.2 ng/mL. ZEN, α-ZEL, β-ZEL, and STC concentrations in the urine are expressed as a ratio to creatinine (pg/mg creatinine).

Both STC and ZEN concentrations in the roughage samples were measured using an API 3200 LC-MS/MS system (AB Sciex, Tokyo, Japan) equipped with an electrospray ionization (ESI) interface and a Prominence HPLC system (Shimadzu Corp., Kyoto, Japan), according to the Food and Agricultural Materials Inspection Center [FAMIC, Food and Agricultural Materials Inspection Center, Japan] at Shokukanken Inc., Gunma, 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, Union, 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 of supernatant was injected into the LC-MS/MS system under the following conditions: column, Synergi 4 μm Polar-RP 80 A (2 mm × 150 mm, 4 μm); oven temperature, 40 ˚C; eluent flow, 200 μL/min; and solvent, methanol (A) + 1 mM Ammonium acetate in 0.1% aqueous acetic acid (B). An ESI probe was used in the positive mode for the STC analysis and the negative mode for the ZEN analysis. The detection limit for each analyte was 0.01 mg/kg. The mean STC and ZEN recovery rates were 90.5%–93.5% and 95.3%–98.5%.

Analytical Methods of AMH and SAA in Serum Samples

Serum AMH concentration was measured using a bovine AMH ELISA kit (AnshLabs, Webster, TX, USA), according to a previous report [Fushimi *et al.,* 2019] to monitor the ovarian AFC of the examined cows during the follow-up period. Briefly, undiluted plasma (50 μL) was used for the assay, which had a limited detection of 11 pg/mL and a coefficient of variation of 2.9%, according to the manufacturer's instructions. Based on our previous studies [Okawa *et al.,* 2021], it is clear that the blood AMH concentration in cattle is lower than usual during the peripartum period; thus, in this study, the AMH concentration in each cow's calving month during the monitoring period was evaluated with particular care. Additionally, SAA concentrations were measured using an automated biochemical analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) with a special SAA reagent for animal serum or plasma (VET-SAA 'Eiken' reagent; Eiken Chemical Co. Ltd., Tokyo, Japan) to monitor the inflammation status of each cow during

sampling. The SAA concentration was calculated using a standard curve generated using a calibrator (VET-SAA calibrator set; Eiken Chemical Co. Ltd., Tokyo, Japan).

Data Management and Statistical Analysis

Monthly estimates for ZEN, AMH, and SAA were calculated using mixed model analysis with subject as a variable factor, because they contain missing data due to calving of the examined cows. Because the ZEN and SAA values approximate a lognormal distribution, the geometric mean estimate was calculated. The AMH value approximates a normal distribution; therefore, the arithmetic mean estimate was calculated. The effects of ZEN and AMH values were evaluated by calculating the simple regression of ZEN values with AMH and the time-lagged regression, which examines the effect of ZEN values one month earlier (lag 1 month), using a linear mixed model. Furthermore, the effects of ZEN on AMH change were evaluated by defining the change in AMH value over one month as the change from the previous month. The analysis was similarly evaluated by calculating the simple regression of ZEN value to AMH change (lag-0 model) and the time-lag regression to examine the effect of ZEN value one month earlier using a linear mixed model. In other words, the lag 1-month model evaluates the effect of the ZEN value of the current month on the AMH change until the next month. In addition, the effect of ZEN on SAA was also evaluated using the same linear mixed model as described above. A two-sided p-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows (version 24.0; IBM Japan, Tokyo, Japan).

All results of the reproductive records of the herds obtained are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using BellCurve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Calving intervals of the herd from 2017 to 2021 were compared using a one-way analysis of variance, followed by a post-hoc test (Tukey-Kramer). Additionally, calving intervals during the pre- (2017 and 2019) and post-ZEN monitoring (2020 and 2021) periods of the examined five cows were compared between the groups using Student's t-test to determine the effects of introducing the monthly urinary ZEN monitoring system on the reproductive efficacies of the breeding herd. Statistical significance was set at $p \leq 0.05$, whereas p-values ranging between 0.05 and 0.1 were considered to indicate a trend toward significance.

RESULTS

First Urinary ZEN Screening on Four JB Breeding Cattle Herds in the Neighborhood

Table 2 shows a summary of all results of the first screening of the four herds. The urinary ZEN concentrations of two samples from Herd C measured by ELISA exceeded the upper limit value that guarantees quantification within the range of the calibration curve used in the ELISA measurement (4,050 ppt = 4,050 pg/mL); thus, they were assumed to be >20,250 pg/mL without repeating ELISA measurements by using more diluted samples, whose concentration levels differed by two orders of magnitude compared to cows from the other three herds. Therefore, based on the results of the first urinary ZEN screening, it was assumed that the ZEN concentration in the roughage fed to herd C was much higher than that in the other herds. Thus, as the next step in our screening, both ZEN and STC concentrations in the dietary roughage from all herds were measured. The ZEN concentration of the roughage sample from Herd C was 1.34 mg/kg, which was higher than the Japanese national limit of ZEN, concomitant with STC exposure (0.08 mg/kg) (**Table 1**). Additionally, the results of LC/MS measurements performed later, i.e., the simultaneous detection of ZEN, its metabolites, and STC in the urine sample only from Herd C, clarified the ELISA results of urine samples and LC-MS/MS results of both ZEN and STC in roughage.

Table 2. Urinary ZEN concentrations at the first screening on four JB breeding cattle herds.

Cow	ELISA		LC-MS/MS					LC-MS/MS	
	Urinary ZEN Concentration (pg/mL)	ZEN/Cre	ZEN/Cre	α-ZEL/Cre	β-ZEL/Cre	ΣZEN/Cre	STC/Cre	ZEN in Roughage (mg/kg)	STC in Roughage (mg/kg)
A1	2,132.60	3,280.90	ND	ND	ND	ND	ND	ND	ND
A2	1,637.60	930.5	ND	ND	ND	ND	ND		
B1	1,937.90	983.7	ND	ND	ND	ND	ND	ND	<0.04**
B2	1,528.50	979.8	ND	ND	ND	ND	ND		
C1	>20,250	>23,011.4*	14,363.60	10,772.70	16,454.50	41,590.90	659.10	1.34	0.08
C2	>20,250	>21,315.8*	11,915.80	8,526.30	4,736.80	25,178.90	442.10		
D1	1,931.20	3,862.4	ND	ND	ND	ND	ND	ND	ND
D2	985.2	1,669.8	ND	ND	ND	ND	ND		

*The urinary ZEN concentrations of the two samples from Herd C ranged over the maximal standard concentration of the ELISA kit. Thus, ZEN/Cre were expressed based on the maximal standard concentrations. Cre: Creatinine.
**Sterigmatocystin was detected below the lower limit, but it was not reached the quantitative value. ND: Not detected.

Follow-up Monthly Monitoring in ZEN Detected JB Breeding Cattle Herd

Sampling could not be performed for Cow 4 in August 2020 because she was about to calve at the time of sampling. The monthly changes in both urinary ZEN concentrations measured by ELISA and serum AMH concentration are shown in **Figure 2**. During the 1-year follow-up period, two peaks of urinary ZEN concentrations were observed in August 2020 and between April and May 2021 with different concentrations in each cow, which were later confirmed by urinary ZEN and metabolite detection by LC-MS/MS measurement (**Figure 2g**). Additionally, STC was also detected in urine samples from four cows in July 2020 (Cow 1: 184.8), February (Cow 4: 508.5), March (Cow 1: 429.4, and Cow 2: 495.7), and one in June 2021 (Cow 1: 342.5) (**Figure 2g**). Alternatively, ZEN was only detected in May 2021 (0.03 mg/kg), and STC was detected in July 2020 (0.01 mg/kg), February (0.02 mg/kg), April (0.03 mg/kg), and May 2021 (0.05 mg/kg), suggesting that feed ZEN exposure does not correspond with urinary ZEN concentrations, and feed STC exposure does not seem to correspond with urinary STC concentrations (**Figure 2h**), which must be due to largely reflected by the influence of the sampling parts collected as roughage samples.

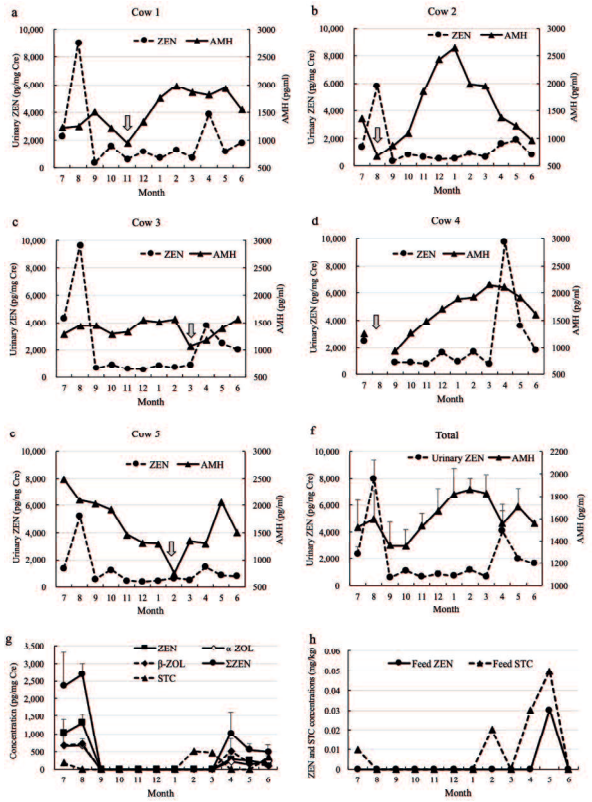

Figure 2. Monthly changes of both urinary ZEN concentration measured by ELISA and serum AMH concentration of each cow; (a) Cow 1, (b) Cow 2, (c) Cow 3, (d) Cow 4, and (e) Cow 5, ↓: calving, (f) total; mean urinary ZEN concentration and AMH from five cows, (g) monthly changes of urinary ZEN, its metabolites, and STC concentrations measured by LC-MS/MS, (h) monthly changes of ZEN and STC concentrations in the dietary roughage measured by LC-MS/MS.

The estimated values of ZEN, AMH, and SAA for each month estimated by linear mixed model analysis are shown in **Table 3** and **Figure 2f**. The ZEN value peaked in August, then decreased from September to March, and trended upward from April. Conversely the AMH value dropped once in September, trended upward in March, then trended downward again in April. The SAA also showed a trend of increasing until March of the following year, although there had been increases and decreases since August.

Table 3. Estimated means and confidence intervals of ZEN, AMH, and SAA at each time point by mixture model.

Date	ZEN		AMH		SAA	
	Geometric Mean	95% CI	Arithmetic Mean	95% CI	Geometric Mean	95% CI
2020/7	2,142.2	1,373.8–3,340.4	1,521.6	1,165.7–1,877.5	2.8	1.5–4.9
2020/8	8,056.5	4,853.3–13,373.8	1,594.5	1,135.0–2,053.9	2.5	1.2–5.2
2020/9	521.1	334.2–812.6	1,358.0	1,002.1–1,713.9	2.9	1.6–5.1
2020/10	1,065.7	683.4–1,661.7	1,356.6	1,000.7–1,712.5	4.0	2.2–7.1
2020/11	627.1	392.9–1,000.9	1,532.9	1,134.9–1,930.8	3.4	1.8–6.4
2020/12	720.8	462.2–1,123.9	1,665.8	1,309.9–2,021.7	3.1	1.7–5.4
2021/1	676.9	434.1–1,055.4	1,820.8	1,464.9–2,176.7	4.9	2.8–8.7
2021/2	995.8	623.9–1,589.5	1,860.0	1,462.1–2,257.9	3.1	1.6–5.8
2021/3	669.9	419.7–1,069.2	1,828.1	1,430.1–2,226.0	5.2	2.8–9.8
2021/4	3,194.8	2,048.8–4,981.8	1,553.8	1,197.9–1,909.7	3.3	1.9–5.9
2021/5	1,763.7	1,131.0–2,750.2	1,704.4	1,348.5–2,060.3	3.0	1.7–5.3
2021/6	1,414.2	885.9–2,257.7	1,554.7	1,156.8–1,952.6	2.2	1.2–4.2

95% CI: 95% confidence interval.

The results of the time-series regression between the ZEN and AMH values are shown in **Table 4**. Although neither correlation was significant, the effect of the ZEN value one month earlier on AMH displayed a negative regression trend ($\beta = -0.449$ [−1.112, 0.214], $p = 0.160$ in lag 1 month model). In other words, a low ZEN value one month prior tended to result in a high AMH value in the current month. The results of the examination of the time-series regression between the ZEN and AMH change values are shown in **Table 5**. Although no correlations were significant, a negative correlation trend was observed for the effect of the ZEN value from one month before AMH change ($\beta = -0.377$, lag 1 month). In other words, a low ZEN value in the current month suggested a tendency for AMH values to be higher in the next month.

Table 4. Regression between ZEN and AMH values.

	AMH		
	β	95% CI	*p*-value
Simple correlation ZEN	−0.085	−0.787 – 0.617	0.793
Time-lagged correlation ZEN (lag 1 month)	−0.449	−1.112 – 0.214	0.160

The effects of ZEN on AMH values were evaluated by calculating the simple regression of ZEN values to AMH and the time-lagged regression, which examines the effect of ZEN values, one month earlier (lag 1 month), using a linear mixed model. β: Standardized regression coefficient. 95% CI: 95% confidence interval.

Table 5. Regression between ZEN and AMH changes.

	AMH Change over one month		
	β	95% CI	*p*-value
Time-lagged correlation ZEN (lag 0 month)	−0.024	−0.744 – 0.695	0.941
Time-lagged correlation ZEN (lag 1 month)	−0.377	−1.039 – 0.285	0.230

The analysis was similarly for Table 4 evaluated by calculating the simple regression of ZEN value to AMH change over one month (lag 0 model) and time-lag regression to examine the effect of ZEN value one month earlier using a linear mixed model.

The results of the time series regression between SAA and ZEN values are shown in **Table 6**. Although both regressions were non-significant, the effect of the current month's ZEN value on SAA showed a negative regression trend (β = −0.400 [−1.046, 0.246], p = 0.198 in lag 0 model). In other words, a high ZEN value may tend to result in a low SAA value in the current month. The results of the examination of the time series regression between SAA change and ZEN value are shown in **Table 7**. All regressions were non-significant, and the regression coefficients were small.

Table 6. Regression between ZEN and SAA values.

	SAA		
	β	95% CI	*p*-value
Simple regression ZEN	−0.400	−1.046 – 0.246	0.198
Time-lagged regression ZEN (lag 1 month)	−0.449	−1.029 – 0.364	0.308

The effects of ZEN on SAA values were evaluated by calculating the simple regression of ZEN values to SAA and the time-lagged regression, which examines the effect of ZEN values, one month earlier (lag 1 month), using a linear mixed model. β: Standardized regression coefficient. 95% CI: 95% confidence interval.

Table 7. Regression between ZEN and SAA changes.

	SAA Change over one month		
	β	95% CI	*p*-value
Time-lagged regression ZEN (lag 0 month)	−0.024	−0.744 – 0.695	0.941
Time-lagged regression ZEN (lag 1 month)	−0.377	−1.039 – 0.285	0.230

The analysis was similarly for Table 6 evaluated by calculating the simple regression of ZEN value to SAA change over one month (lag 0 model) and time-lag regression to examine the effect of ZEN value one month earlier using a linear mixed model.

The calving interval of the herd were 389.8 ± 35.3 (n = 10) in 2018, 471.7 ± 33.1 (n = 18) in 2019, 387.8 ± 15.0 (n = 19) in 2020, and 408.5 ± 24.6 (n = 20) in 2021, and tendency toward decreased calving intervals (p = 0.099) was observed between 2019 (pre-ZEN monitoring period) and 2020 (post-ZEN monitoring period). **Table 8** shows the results of the calving intervals of the five cows examined during the pre- and post-ZEN monitoring periods. The number of calving intervals during the post-ZEN monitoring period (349.9 ± 6.9) was significantly lower (p = 0.0487) than the pre-ZEN monitoring period (439.0 ± 41.2).

Table 8. Mean calving intervals of the examined 5 cows during pre- and post-ZEN monitoring periods.

	Birthday	Pre-monitoring			Post-monitoring	
		2017 (Pre)	2018 (Pre)	2019 (Pre)*	2020 (Post)**	2021 (Post)
Cow 1	9/Jan/2016	-	351	335	349	333
Cow 2	8/Nov/2014	690	-	380	321	349
Cow 3	7/Apr/2014	346	392	437	334	346
Cow 4	15/Jul/2015	-	600	-	377	355
Cow 5	27/Dec/2016	-	-	420	-	385
Mean of 5 cows		518.0 ± 172.0	447.7 ± 77.1	393.0 ± 22.7	345.3 ± 12.0	353.6 ± 8.6
Mean of the pre- and post-monitoring		439.0 ± 41.2[a] (n = 9)			349.9 ± 6.9[b] (n = 9)	

*Pre: Pre-ZEN monitoring period, **Post: Post-ZEN monitoring period. a,b: $p < 0.05$.

DISCUSSION

Currently, many reports have aimed to clarify and prevent the harmful effects of mycotoxins at each stage focused on three major factors. First, the characters, toxicity, and metabolites against the organs and/or systemic function of each mycotoxin (including emerging mycotoxins such as enniatin, beauvericine, and emodin) with both in vitro and in vivo approaches [Reisinger *et al.,* 2019; Kinkade *et al.,* 2021]. Second, the detection methods for these mycotoxins (including the case of multiple mycotoxins coexistence with different *spp.* of fungi) within dietary feeds and biological fluids, such as serum, urine, and milk, from animals [Lee *at al*., 2018; Vandicke *et al.,* 2019; Panasiuk *et al.,* 2019; Nualkaw *et al.,* 2020]. Third, not only feed management and control strategies for fungal infection but also the degradation approaches by physical, chemical enzymatic, and biological methods to prevent the harmful effects of mycotoxins for the animals [Liew *et al.,* 2018; Li *et al.,* 2020]. In cattle practice, acute exposure to high doses of mycotoxins is usually responsible for well-characterized clinical symptoms, such as reduced feed intake or diarrhea. Sub-chronic and chronic exposure to low doses has been less well characterized but is considered to be responsible for reduced performance, for reduced pathogen resistance, and more generally, for many of the causes of damaged health, and potentially the reproductive efficacy, of the herd [Guerre *et al.,* 2020]. Therefore, it is essential to first monitor the contamination status of mycotoxins in dietary feeds at each farm level to limit the exposure risks of mycotoxins. As previously suggested, one practical approach is to evaluate the feed contamination on each farm with an ELISA test kit for mycotoxin screening, followed by further validation of the suspected feed samples with LC-MS/MS [Liu *et al.,* 2020]. Following these approaches, one objective of the present field trial/test was to 1) evaluate and apply the urinary ZEN monitoring system for its practical usefulness in cattle farm conditions. As expected, the results of our first screening indicated that (1) urinary ZEN measurements may be useful for monitoring or evaluating the level of intestinal absorption of ZEN from dietary feeds with follow-up by even small urinary samples (0.5 mL) from the same herd, (2) it was possible to detect ZEN naturally contaminated cattle herds by relatively high ZEN levels in feeds by ELISA as a rather simple method within the laboratory, concomitant with the coexistence of STC contamination of the dietary rice straw or WCS by following LC-MS/MS measurement, and (3) in a cattle herd (C) with confirmed ZEN exposure, monthly monitoring in the following year made it possible to monitor and control the exposure levels of dietary roughages derived from rice straw from within the same paddy

field. To the best of our knowledge, this is the first practical verification test conducted in some cattle in which data are available encompassing the period from detection to follow-up using the urinary ZEN monitoring system.

The greatest advantage of using the urinary ZEN concentration monitoring system is that the ZEN concentration that is actually ingested and absorbed from the intestinal tract can be monitored and compared with other herds. As previously reported [Bryden *et al.,* 2012; Fushimi *et al.,* 2014], the problem is that the concentration of mycotoxins produced in dietary feeds may vary greatly depending on the collection site of the feed sample to be collected. Indeed, in the present study, different results between the urinary ZEN concentration and the ZEN concentration in the roughages in August 2019 seem to clearly show this problem. Using the urinary ZEN monitoring system, it is possible to monitor the concentration of mycotoxins absorbed from the intestinal tract, and the absorbed concentration of mycotoxins may reflect the degree of contamination of the mycotoxins in the feed for each herd and its feed intake by animals. Since urinary ZEN concentration may be affected by the intake volumes of contaminated feeds, it seems to be a suitable method for monitoring and comparing mycotoxin exposure in cattle whose daily feed amount is fixed between each herd. In fact, in this study, although the urinary ZEN concentrations of the two heifers in Herd D (3862.4 and 1669.8) were like those of Herd A (3280.9 and 930.5), the urinary concentration was approximately 2 to 4 times higher than that of Herd B (983.7 and 979.8). Presumably, when comparing the daily feed volume, especially roughage, amounts for cows in Herd D were approximately half that of cows in Herd B. Naturally, the ZEN contamination level was lower than that of cows in Herd C, in which ZEN exposure was detected that time. However, in terms of the level of natural contamination of ZEN in roughage, that for cows in Herd D was higher than for cows in Herds A and B because the daily roughage feed for herd D was half that of Herds A and B. This demonstrates again that it can be inferred by performing ZEN monitoring to compare contamination levels within the rice straw in the present study. To investigate the effects of chronic mycotoxin exposure on the health status and productivity of livestock herds, a urinary mycotoxin monitoring system for monitoring mycotoxin intake from dietary feeds is indispensable. As we have demonstrated in this study, the simultaneous screening of cattle herds in the same area with similar breeding environments will be an important future strategy to understand the status of mycotoxin contamination of cattle herds. Additionally, the measurement results of ELISA and LC-MS/MS, the two measurement methods used for ZEN concentration measurement in this study, indicate that the urinary ZEN measurement using the ELISA method is an

accurate, simple, and useful measurement method for evaluating the dynamics of mycotoxin infiltration at rather low concentrations and long-term chronic exposure.

The second purpose of the present study was to evaluate both ZEN (*Fusarium* mycotoxin called pre-harvest mycotoxin) and STC (*Aspergillus* mycotoxin called post-harvest mycotoxin) dynamics in the dietary roughage (rice straw and WCS) using the naturally ZEN (also STC) contaminated herd (Herd C) detected in August 2019 as a model/examined herd, mainly by urinary ZEN monitoring during the year from July 2020 to June 2021. In addition, ZEN and its metabolites have been suggested to cause apoptosis of granulosa cell/atresia of follicles in several animals [Minervini *et al.,* 2006; Zhu *et al.,* 2012; Zhang *et al.,* 2018; Li *et al.,* 2020], the relationship between urinary ZEN and AMH concentration during the monitoring period was studied to clarify the effects of ZEN exposure on AMH secretion from antral follicles. In this regard, similar to our recently reported decrease in AMH concentration during the peripartum period [Okawa *et al.,* 2021], all five examined JB cows displayed a clear decline in AMH concentrations in the month of their calving with a large range of SAA concentrations. Thus, we deleted all AMH and SAA concentration data for the calving month of each cow from our data set in the present study. As a result, although it became clear that there was a large variation in AMH concentration in each month among each individual cow during 1 year period in the blood samplings (**Figure 2a–e**), our results regarding the relationship between ZEN and AMH suggest that natural exposure level of ZEN may affect AMH concentrations, and thus, the AFC in cattle ovaries (**Figure 2f, Tables 4 and 5**). Our results indicated that a low ZEN value one month prior may tend to result in a high AMH value in the current month, and a low ZEN value in the current month suggested a tendency for higher AMH values in the next month. Therefore, it was suggested that when AMH rises, it may be affected by the ZEN value of the previous month, and when AMH decreases, it may be affected by the ZEN value of the current month. Thus, the effects of ZEN on AMH secretion appeared early but recovery of AMH secretion after ZEN exposure may take some time. As an interesting result obtained from the present study, a negative regression trend between the concentrations of ZEN and SAA; a high ZEN value may tend to result in a low SAA value in the sampling month, were observed (**Tables 6 and 7**). ZEN has been reported to have immunotoxicity in addition to its endocrine disrupting effects [Pistol *et al.,* 2014; Bulgaru *et al.,* 2021]. Previous reports indicated that ZEN exposure altered the hepatic cellular immune response, and suppressed the secretion of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α [Pistol *et al.,* 2014; Bulgaru *et al.,* 2021; Lee *et al.,* 2021]. Therefore, the negative regression trend between urinary ZEN

and SAA concentrations obtained in the present study is possibly due to innate immune suppression of cows by low-level chronic ZEN exposure. In the future, it will be necessary to increase the number of cow herds monitored, expand the scope of monitoring, and clarify that improving the feed while detecting the naturally exposed herd will lead to an improvement in productivity. At the same time, field tests in the process of improving the mycotoxins level in naturally contaminated feed will be important indicators of animal health risks.

Several incidences of STC contamination in food and feed (e.g., grains, grain-based products, maize, and rice) have also been reported in Japan [Kobayashi *et al.,* 2018, 2019; Nomura *et al.,* 2018; Yoshinari *et al.,* 2019]. Rice straw is considered one of the most important roughages used in the production of beef cattle in Japan, and STC is a major mycotoxin produced in rice. However, the harmful or chronic effects of STC on cattle are not well understood, and there are no regulations or control measures for this toxin in Japan. Previous measures of large-scale in-feed mycotoxins confirm a large difference in the types of mycotoxins when multiple mycotoxins were detected in feed coexist in each country and region of the country [Gruber-Dorninger *et al.,* 2019]. In the present study, ZEN and STC co-exposure in rice straw (WCS) was also confirmed in the area screened, another prefecture in the Kyushu area where we previously detected co-exposure to ZEN and STC. Our results elucidate the characteristics of mycotoxin co-contamination of rice straw produced in Japan and future research should further expand the scope of the survey to understand the characteristics and relationships between the two mycotoxins.

ZEN and its metabolites exhibit distinct estrogenic properties that affect the reproductive system of several animal species, especially pigs [Kleinova *et al.,* 2002; Fink-Gremmels *et al.,* 2007; Minervini *et al.,* 2008]. In contrast, clinical signs of hyperestrogenism are not frequently observed in ruminating cows, and then only following the ingestion of highly contaminated silage or long-term exposure to contaminated feed materials [Weaver *et al.,* 1986[a,b]; Fink-Gremmels, 2008]. In the present study, we simply compared the calving intervals of the monitored herd before and after introducing the urinary ZEN monitoring system and observed significantly reduced calving intervals of the herd. We previously reported the in vitro effects of acute ZEN exposure on bovine oocytes by using in vitro maturation, in vitro fertilization (IVF), and in vitro culture systems in cattle, and found that a high ZEN concentration (>1 mg/kg 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 reported that

natural-feed ZEN contamination levels below the threshold value (i.e., below the maximum permissible ZEN concentration in Japan) did not affect embryo production in Japanese Black and Holstein cows undergoing superovulation [Takagi *et al.,* 2013]. Therefore, it was suggested that ZEN-contaminated feed affects the fertility of cattle by influencing the development of embryos in the uterus after implantation. In this study, the roughages harvested in 2019, which were fed prior to our first ZEN screening, were ZEN-contaminated, and the long-term use of the contaminated feed affected the calving interval. It is speculated that the introduction of the urinary ZEN monitoring system controlled ZEN contamination in the feeds, which shortened the calving interval of the herd. Obviously, further studies with an increased number of monitor herds in the field are needed.

In conclusion, our results demonstrate that the urinary ZEN monitoring system is an important practical tool, not only for detecting contaminated herds under field conditions but also for revealing the effects of long-term chronic ZEN/STC (or other co-existing mycotoxins) exposure on herd productivity and fertility. To date, several approaches have been developed to reduce mycotoxin contamination and exposure, including strategies involving agronomy, plant breeding and transgenics, biotechnology, toxin binding, and deactivating feed additives, and feed supplier/animal producer education [Bryden *et al.,* 2012]. As shown in the present field trial, herd management with a urinary ZEN monitoring system may be a possible novel concept for creating awareness among herd managers thereby preventing mycotoxin exposure in cattle herds.

Chapter 2

Practical application of a urinary zearalenone monitoring system for feed hygiene management of a Japanese black cattle breeding herd— relevance to anti-Müllerian hormone and serum amyloid A clarified from a two-year survey

ABSTRACT

In this study, a herd of Japanese Black (JB) breeding cattle with sporadic reproductive disorders was continuously monitored for an additional year to assess the effects of the urinary zearalenone (ZEN) concentration and changes in parameters (AMH and SAA) with time-lag variables and herd fertility (reproductive performance). This herd had high (exceeded the Japanese dietary feed regulations) urinary ZEN and rice straw ZEN concentrations (1.34 mg/kg). Long-term data of the herd with positive ZEN exposure revealed a decreasing ZEN concentration in urine and a gradual decrease in the AMH level with age. The AMH level was significantly affected by the ZEN value 2 months earlier and the AMH level in the previous month. The changes in ZEN and SAA values were significantly affected by the ZEN and SAA values in the previous month. Additionally, calving interval data between pre-monitoring and post-monitoring showed a significantly different pattern. Furthermore, the calving interval became significantly shorter between the time of contamination (2019) and the end of the monitoring period (2022). In conclusion, the urinary ZEN monitoring system may be a valuable practical tool for screening and detecting herd contamination in the field, and acute and/or chronic ZEN contamination in dietary feeds may affect herd productivity and the fertility of breeding cows.

INTRODUCTION

Zearalenone (ZEN), an estrogenic mycotoxin from *Fusarium* species that can disrupt cattle reproductive physiology, is one of the most commonly identified mycotoxins in grains and animal feed [Gruber-Dorninger *et al.,* 2019; Eskola *et al.,* 2019]. Several studies reported mycotoxin contamination in feed by detecting the presence of ZEN [Rodrigues *et al.,* 2011; Streit *et al.,* 2012; Jalilzadeh-Amin *et al.,* 2023; Muñoz-Solano and González-Peñas, 2023; Garcia *et al.,* 2023]. Despite efforts to minimize mycotoxin concentrations in animal feeds, the prevalence of *Fusarium*-derived mycotoxins is increasing worldwide. ZEN is not degraded by feed processing such as milling, extrusion, storage, and heating (thermally stable) [Gromadzka *et al.,* 2008; Abbes *et al.,* 2020]. Therefore, monitoring and detecting ZEN contamination is important [Castelló *et al.,* 2022]. The adverse effects of mycotoxins on reproductive performance should be minimized by managing and caring for livestock to avoid mycotoxin contamination. Zearalenone contamination in herds can be assessed by measuring urinary ZEN metabolites in selected cows because it is absorbed in the gastrointestinal tract and excreted in urine after hepatic metabolism. Previously, we established a urinary ZEN monitoring system in cattle using ELISA for initial screening purposes, followed by LC-MS/MS validation to detect ZEN and its related metabolites [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012; Widodo *et al.,* 2022]. We also evaluated the efficacy of mycotoxin adsorbents (MAs) added to the diet to reduce the intestinal uptake of mycotoxins [Takagi *et al.,* 2011].

Building on our previously published study [Widodo *et al.,* 2022], we applied our monitoring system on Japanese Black (JB) breeding cattle herds and reported the usefulness of the system to detect a ZEN-contaminated herd, and the possible relationship among the parameters such as ZEN, anti-Müllerian hormone (AMH; a potential predictor of fertility, superovulation response, ovarian disorders, and herd longevity [Mossa *et al.,* 2017, 2019; Umer *et al.,* 2019; Ramesha *et al.,* 2022]), and serum amyloid A (SAA; known as an acute-phase protein, and a sensitive and not only early indicator of inflammation but also an effective diagnostic aid in animal reproduction [Chan *et al.,* 2010; Eckersall *et al.,* 2010; Bazzano *et al.,* 2022]) concentrations based on their monthly measurements of fixed five cows during the 1-year follow-up ZEN contamination mitigation period under a ZEN-affected herd. As emphasized in our previous report [Widodo *et al.,* 2022], we believe that long-term monitoring of spontaneous (naturally occurring) cases is key to clarifying the

effects of dietary mycotoxin contamination on cattle health, especially the reproductive efficacy of breeding cattle herds. We continued monitoring for an additional year after the last report [Widodo *et al.,* 2022], providing monthly guidance on feeding management to farmers and confirming the effectiveness of countermeasures based on the results of two years of follow-up.

In this communication, we report the follow-up results obtained from our long-term urinary ZEN monitoring system in a JB cattle herd from July 2020 to July 2022. Here, we analyzed the effects of long-term (2 years) relationships among the ZEN-AMH-SAA associations obtained from our monthly on-farm follow-up measurements, and how the reproductive efficacies improved following our consultation with the farmers based on the results of the monthly ZEN-AMH-SAA measurements, whose relationship could not be clarified from only the one-year results alone, but was clarified using the two-year data in this herd.

MATERIAL AND METHODS

All experiments were conducted in accordance with the Guidelines and Regulations for the Protection of Laboratory Animals and the guidelines of Yamaguchi University (No. 40 of 1995, approved on 27 March 2017). Informed consent was obtained from all participants.

ZEN Monitoring and Reproductive Performance Evaluation

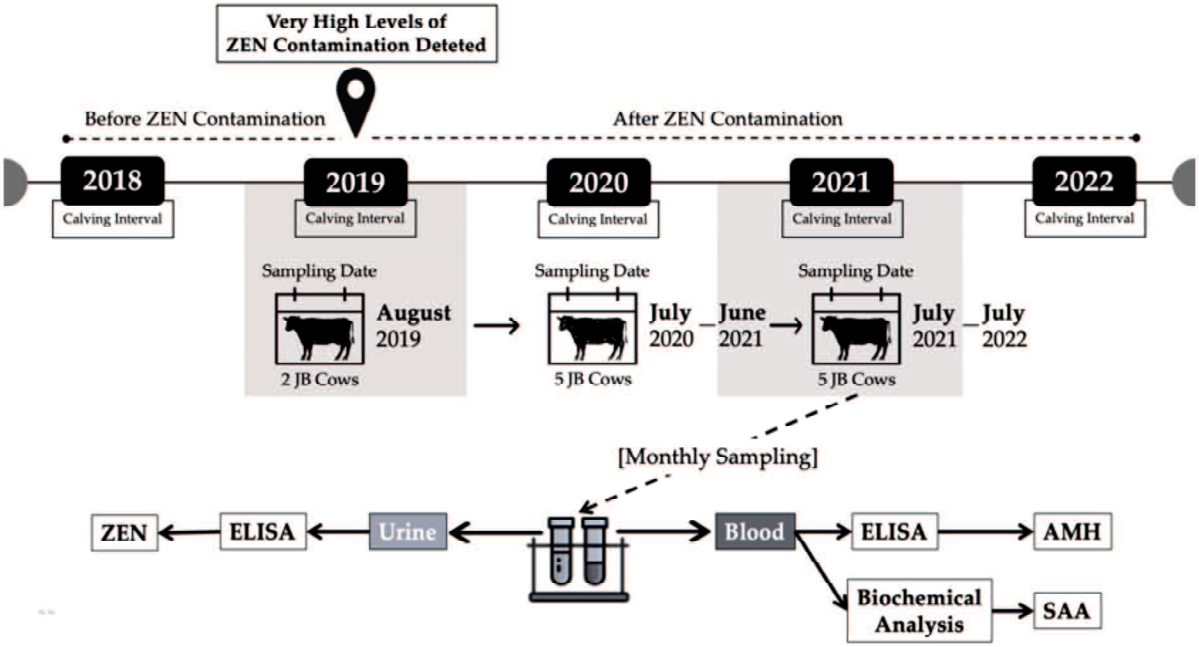

Figure 1. Schematic diagram of experimental design. ZEN: zearalenone; AMH: anti-Müllerian hormone; SAA: serum amyloid A.

After the detection of very high zearalenone contamination (in urine: >20,250 pg/mL and feed: 1.34 mg/kg) in August 2019, to maintain a similar situation in the following year, the first monitoring of the herd was performed from July 2020 to June 2021 [Widodo *et al.,* 2022]. The monitoring research of the first year was published. To determine the condition of the herd, a second year of monitoring was performed from July 2021 to July 2022 (long-term monitoring, first and second years). Based on the first-year monitoring evaluation, only the ZEN concentrations in urine samples were analyzed in the second year. Five cows from the same feeding and rearing management group were sampled routinely at the beginning of each month, approximately 2 hours after the morning feeding. The body weight of the cows was approximately 500 kg, with an age range of 4–7 years. Generally, fresh urine was collected by massaging or stimulating the area surrounding the outer vulva. Approximately 10 mL of fresh urine was collected and stored in a tube, and sample identities were recorded.

Fresh urine was stored immediately in a refrigerated sample box and transported to the laboratory. After centrifugation (3,500 rpm for 5 min), urine samples were placed in microtubes (approximately 1.5 mL) and stored in a −30 °C freezer until sample analysis was performed. ZEN concentrations were performrd every two months using ELISA. The detailed analytical methods are described in the next subsection. Calving intervals were referenced and compared annually from 2018 to 2022 as the reproductive records. This study was conducted to verify the efficacy of a monthly ZEN monitoring system. Between 2018 and 2022, there were 10, 18, 19, 20 and 20 parturitions from 24 cows in the herd. A schematic representation of the study design is shown in **Figure 1**.

Analysis Methods of ZEN, AMH, and SAA

The concentration of ZEN in urine was determined using a commercially available kit (RIDASCREEN® Zearalenon; R-Biopharm AG, Garmstadt, Germany) according to the manufacturer's instructions with minimal modifications. In brief, a urine sample (0.1 mL:5-fold kit dilution) was added to 3 mL of 50 mM sodium acetate buffer (pH 4.8), and the solution was incubated for 15 h at 37 °C in the presence of 10 μL of β-glucuronidase/arylsulfatase solution. The samples were then placed on a C18 solid-phase extraction (SPE) column (Strata; Phenomenex, Torrance, CA, USA) preconditioned with 3 mL of methanol, followed by 2 mL of 20 mM Tris buffer (pH 8.5) and methanol (80:20). The SPE column was washed with 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20) and 3 mL of 40% methanol before drying by centrifugation (500 × *g*) for 10 min. The analytes were gently eluted (flow rate: 15 drops/min) using 1 mL of 80% methanol. Using a centrifugal evaporator, the eluate was evaporated to dryness at 60 ◦C. The dried residue was redissolved in 50 μL of methanol, followed by 450 μL of sample dilution buffer, properly mixed, and an aliquot of 50 μL was utilized for the ELISA experiment. A RIDA®SOFT Win (R-Biopharm, Art. No. Z9999) was used to determine the absorbance at 450 nm using a microplate spectrophotometer to quantify the ZEN concentration in urine samples. Urine creatinine concentrations were assessed using a commercial kit (Sikarikit-S CRE; Kanto Chemical, Tokyo, Japan) and quantified using a 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan) according to the manufacturer's instructions. All urine values were given as creatinine ratios (pg/mg creatinine) according to a previous report [Hasunuma *et al.,* 2012].

To monitor the ovarian antral follicle count (AFC) of the examined cows over the long-term monitoring period, we determined the serum AMH concentration using a bovine

AMH ELISA kit (AnshLabs®, Webster, TX, USA). In brief, 50 µL of undiluted plasma was added to 50 µL of AMH assay buffer. After 2 hour, the cells were incubated and shaken (600–800 rpm) in an orbital microplate shaker. An incubation temperature of 23 ± 2 °C yielded the best results. We aspirated and washed with wash solution (5 times) after incubation, then added the AMH Antibody-Biotin Conjugate-RTU (100 µL), incubated, and shook (600–800 rpm) for 1 hour. We washed the solution, added 100 µL of AMH Streptavidin-Enzyme Conjugate-RTU, and incubated for 30 min. Afterward, we washed once more, added 100 µL of TMB chromogen solution, incubated, and shook (600–800 rpm) for 10–12 min. Finally, we added the stopping solution (100 µL) and measured the absorbance of the solution within 20 min with a 450 nm microplate reader. According to the manufacturer's instructions, the analytical sensitivity and imprecision were 11 pg/mL and 2.92% (coefficient of variation), respectively.

In addition, SAA concentrations were determined during sampling using an automated biochemical analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) and a particular SAA reagent for animal serum or plasma (VET-SAA 'Eiken' reagent; Eiken Chemical Co. Ltd., Tokyo, Japan). The SAA concentration was calculated using a calibratorgenerated standard curve (VET-SAA calibrator set; Eiken Chemical Co., Ltd., Tokyo, Japan).

Feeding Management

The cows in this herd were subjected to the same feed management. Feed is administered to pens as separate forage (79.5%) and concentrate (20.5%) [Widodo *et al.,* 2022]. Forage feeds consist of home-grown rice straw, 12–14 kg (71.8%), and orchard grass, 10 kg per week (7.7%); formula feeds consist of commercially available concentrates, 3 kg (15.4%), and wheat, 0.5–1 kg (5.1%). Orchard grass was provided only once a week. The concentrate administered to cows was a factory-made commercial concentrate tested for mycotoxin contamination. Feed was assessed based on routine monthly monitoring of ZEN contamination in urine. When a high level of ZEN contamination was identified in urine, we promptly coordinated and discussed the necessity of replacing the feed with veterinarian management and farmers. The presence of ZEN in urine may indicate ZEN contamination in cow feed.

Data Management and Statistical Analysis

Before proceeding with the vector autoregression (VAR) model, the null hypothesis was evaluated using the Phillips–Perron test. A multivariate time-series study analyzed the monthly estimates of ZEN, AMH, and SAA using the VAR model. The VAR model is a multivariate time-series model that compares current observations of a variable to previous measurements of that variable and other variables in the system and examines the effect of ZEN, AMH, and SAA values from one (lag 1-month) and two months earlier (lag 2-month). In addition, the effects of the changes in ZEN, AMH, and SAA were evaluated by defining the monthly value change (Δ) as the change from the previous month. We examined ΔZEN, AMH, and SAA for their effects on 1- and 2-month lags. Based on the results of the analysis, a p-value ≤ 0.05 indicated a significant result. All statistical analyses were performed using the R Project for Statistical Computing, version 4.2.2.

The Mann–Whitney U test (a non-parametric statistical test) was used to determine the normality of the calving interval data as a reproductive record. We performed statistical tests on the 2019 and 2020 calving interval data until 2022 to compare the calving intervals at that time and after the occurrence of contamination. Finally, we compared the differences in the calving intervals when contamination occurred in 2019 and at the end of the monitoring period in 2022. A p-value ≤ 0.05 indicates a significantly different result.

RESULTS

Long-Term (2 Years) Monitoring of ZEN in a JB Breeding Cattle Herd

We sampled urine and blood monthly for two years, from July 2020 to July 2022. The data for the first half of the year have been previously reported [Widodo *et al.,* 2022]. In this communication, the data for the second half of the year and those over the two years of study are reported. We used a monthly sample of five cows in this study. In February 2022, a new cow was added to replace another cow that would be culled in the following month. Thus, the total number of cows in that month was six. From March 2022 to the end of this study, the number of cows returned to normal (five cows), and a new cow from the same herd was included. Monthly changes in both urinary ZEN and serum AMH concentrations are shown in **Figure 2**. Previously, it was reported that there were two peaks of urinary ZEN contamination detected during monitoring in August 2020 and April 2021 [Widodo *et al.,* 2022]. Subsequently, the herd's urinary ZEN contamination was maintained at a low and steady level. Overall, monitoring of urinary ZEN contamination from July 2020 to July 2022 revealed a declining trend, as indicated by the red dotted line. Similar to the urinary ZEN concentration, the serum AMH level showed a decreasing trend, as indicated by the blue dotted lines. The mean, highest, and lowest AMH values ± SEM were 1431.4 ± 39.07, 1820.8 ± 228.14, and 1083 ± 176.41 pg/mL, respectively.

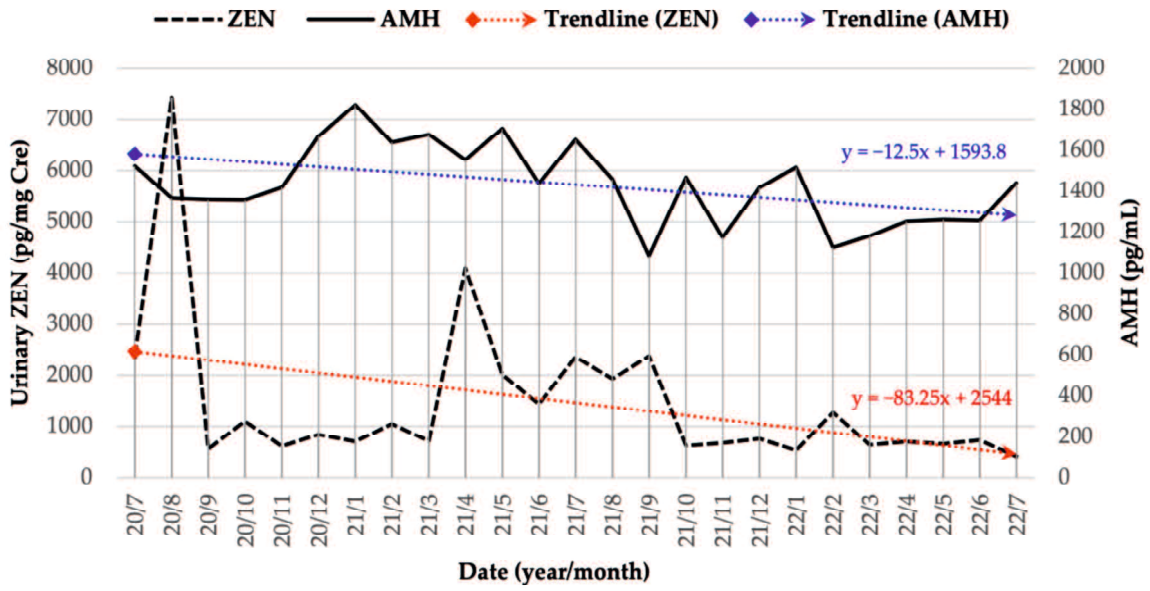

Figure 2. Monthly changes in both urinary ZEN and serum AMH concentrations for 2-year period, mean values of all cows, and linear performance trendlines. ZEN: zearalenone; AMH: anti-Müllerianian hormone; y: trend line equation.

The results of the vector autoregression (VAR) model between ZEN, AMH, and SAA

Values (second-year data) and ZEN, AMH, and SAA changes (second-year data) are shown in **Tables 1 and 2**. The results of the VAR model between ZEN, AMH, and SAA changes (second-year data) could not be statically analized until two months earlier. Only data from the previous month were obtained. Monthly monitoring, which was carried out for only one year, did not indicate a definite association, as our earlier data did [Widodo *et al.,* 2022]. Therefore, to obtain a straightforward interpretation, long-term monitoring was conducted for 25 months to obtain a total of 2 years of monthly monitoring data. The results of long-term monitoring of the VAR model between the ZEN, AMH, and SAA values and ZEN, AMH, and SAA changes sequentially are shown in **Tables 3 and 4**.

Table 1. Vector autoregression between ZEN, AMH, and SAA values (2nd-year data)

	ZEN			AMH			SAA		
	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value
ZEN (lag 1-month)	–0.227	0.401	0.611	0.336	0.171	0.145	0.001	0.002	0.238
ZEN (lag 2-month)	0.545	0.229	0.098	–0.247	0.098	0.086	0.001	0.001	0.094
AMH (lag 1-month)	1.116	0.833	0.272	0.214	0.356	0.590	–0.002	0.004	0.646
AMH (lag 2-month)	2.021	0.825	0.092	0.640	0.353	0.167	0.002	0.004	0.710
SAA (lag 1-month)	–61.186	54.531	0.344	5.901	23.325	0.817	–0.603	0.274	0.115
SAA (lag 2-month)	–54.915	39.886	0.262	35.217	17.061	0.131	0.350	0.201	0.179

Std. Error: standard error. (lag 1-month): one month earlier. (lag 2-month): two months earlier

Table 2. Vector autoregression between ZEN, AMH, and SAA change (2nd-year data)

	ΔZEN			ΔAMH			ΔSAA		
	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value
ΔZEN (lag 1-month)	–0.220	0.463	0.652	0.188	0.177	0.331	–0.001	0.002	0.600
ΔAMH (lag 1-month)	0.699	1.198	0.581	–0.142	0.459	0.768	–0.008	0.005	0.184
ΔSAA (lag 1-month)	–63.11	62.435	0.351	–9.712	23.925	0.699	–0.455	0.283	0.159

Δ: the difference between months or the change in value

The results of long-term monitoring data analysis revealed a significant correlation. Considering the ZEN, AMH, and SAA values comprehensively as shown in **Table 3**, the AMH level was strongly affected by ZEN in the previous two months (p = 0.049) and the AMH level itself in the previous month (p = 0.007). The ZEN value of the previous two months showed a negative trend (estimate = −0.042), whereas the AMH value in the previous month showed a positive trend (estimate = 0.41) based on the current AMH value. In other words, a low ZEN value in the previous two months might have caused an increase

in the AMH level in the current month, and a low AMH level in the previous month may indicate a low AMH level in this month, and vice versa.

Table 3. Vector autoregression between ZEN, AMH, and SAA value (long-term monitoring data)

	ZEN			AMH			SAA		
	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value
ZEN (lag 1-month)	5.75×10^{-3}	1.30×10^{-1}	0.965	–0.012	0.021	0.582	-1.48×10^{-5}	2.66×10^{-4}	0.956
ZEN (lag 2-month)	-6.12×10^{-2}	1.27×10^{-1}	0.632	–0.042	0.021	0.049 *	3.01×10^{-4}	2.59×10^{-4}	0.252
AMH (lag 1-month)	9.98×10^{-1}	8.80×10^{-1}	0.264	0.410	0.144	0.007 **	-1.47×10^{-3}	1.80×10^{-3}	0.420
AMH (lag 2-month)	1.16×10^{0}	9.21×10^{-1}	0.216	0.299	0.151	0.054	3.58×10^{-3}	1.89×10^{-3}	0.065
SAA (lag 1-month)	1.53×10	7.20×10	0.833	1.814	11.759	0.878	5.49×10^{-3}	1.47×10^{-1}	0.711
SAA (lag 2-month)	-1.02×10^{2}	7.26×10	0.170	12.499	11.859	0.298	2.38×10^{-1}	1.49×10^{-1}	0.117

*: *p*-value = 0.01—< 0.05, **: *p*-value = 0.001—< 0.01

In **Table 4**, the change (Δ) in the AMH value was significantly affected by the previous two months' ΔZEN (p = 0.016) and the ΔAMH value itself in the previous month (p = 0.005). Based on the current Δ AMH value, the ΔZEN value for the previous two months (estimate = −0.045) and the ΔAMH value for the previous month (estimate = −0.428) both revealed a negative trend. In other words, a low ΔZEN value in the previous two months might lead to an increase in the current month's ΔAMH value, and a low ΔAMH value in the previous month could lead to a high ΔAMH value this month, and vice versa. Further results demonstrated that the changes in the values of ZEN and SAA were strongly influenced by their values from the previous month (p = 0.003 and p = 0.002, respectively). Both indicated a downward trend (estimate = −0.441 and −0.527), which implies that if the Δ ZEN or ΔSAA value was high during the previous month, the Δ ZEN or Δ SAA value in the following month would decrease, and vice versa. **Table S1**, as supplementary materials, provides supporting information on the ZEN, AMH, and SAA measurement results.

Table 4. Vector autoregression between ZEN, AMH, and SAA change (long-term monitoring data)

	ΔZEN			ΔAMH			ΔSAA		
	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value	Estimate	Std. Error	*p*-value
ΔZEN (lag 1-month)	–0.441	0.137	0.003 **	–0.003	0.019	0.855	2.3×10^{-5}	2.7×10^{-4}	0.933
ΔZEN (lag 2-month)	–0.242	0.129	0.067	–0.045	0.018	0.016 *	3.5×10^{-4}	2.6×10^{-4}	0.179
ΔAMH (lag 1-month)	1.725	1.049	0.109	–0.428	0.145	0.005 **	–0.002	0.002	0.266
ΔAMH (lag 2-month)	1.662	1.064	0.126	–0.199	0.147	0.184	0.002	0.002	0.475

ΔSAA (lag 1-month)	27.007	80.606	0.739	–7.057	11.111	0.529	–0.527	0.161	0.002 **
ΔSAA (lag 2-month)	–70.452	81.193	0.391	5.148	11.192	0.648	0.057	0.163	0.728

*: *p*-value = 0.01—< 0.05, **: *p*-value = 0.001—< 0.01

Finally, based on the findings of the statistical analysis with 2 years of monitoring, we developed formulas to calculate the estimated value of AMH and the change values of AMH, ZEN, and SAA.

Established new formulas:

$$AMH = 0.4 \times AMH_{\text{Last month}}^{**} - 0.04 \times ZEN_{\text{Last 2 month}}^{*} + 434^{*}$$

$$\Delta AMH = -0.4 \times \Delta AMH_{\text{Last month}}^{**} - 0.04 \times \Delta ZEN_{\text{Last 2 month}}^{*} - 9$$

$$\Delta ZEN = -0.4 \times \Delta ZEN_{\text{Last month}}^{**} - 34\ \Delta SAA = -0.5 \times \Delta SAA_{\text{Last month}}^{**}$$

Description of *: *p*-value = 0.01—<0.05, **: *p*-value = 0.001—<0.01. R squared (R^2) in the AMH, ΔAMH, ΔZEN, and ΔSAA formulas is defined as follows: 0.45, 0.37, 0.3, and 0.4.

Calving Intervals as a Reproductive Indicator

The calving interval (days) statistics for 2018, 2019, 2020, 2021, and 2022 are provided in the following order (mean ± SEM): 389.8 ± 35.3; 471.1 ± 33.1; 387.8 ± 15; 408.5 ± 24.6; 368 ± 10. Pre-monitoring data from 2019 were compared with post-monitoring data. **Figure 3** shows a decreasing trend from 2019 to 2022, indicating that the calving interval is shortening. The R squared (R^2) was 0.69 (69%), suggesting that the year variable (urinary ZEN monitoring) had an effect of 69% on the day variable (calving interval). The remaining component (31%) was affected by factors outside this regression equation or variables not investigated. Finally, calving intervals of the cattle herds were compared before and post-ZEN monitoring. Pre-ZEN monitoring was performed in 2019 and post-ZEN monitoring in 2020, 2021, and 2022, with final results after the last year of ZEN monitoring completed in 2022, as shown in **Table 5**. The number of calving intervals during the post-ZEN monitoring periods ((388.2 ± 10.3) and (368.3 ± 10)) was significantly lower (p = 0.007 and p = 0.005) than that of the pre-ZEN monitoring period (471.7 ± 33.1).

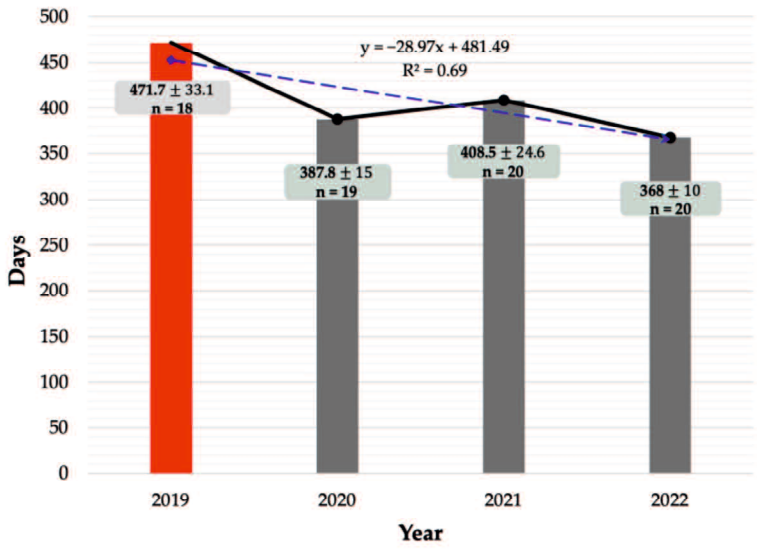

Figure 3. The herd's calving interval during contamination in 2019 (pre-ZEN monitoring) and after contamination in 2020, 2021, and 2022 (post-ZEN monitoring), mean values, SEM, and annual number of births. SEM: standard error of mean; n: annual number of births; R^2: R squared; y: trend line equation.

Table 5. Group statistics on the calving interval between pre- and post-ZEN monitoring

Pre-monitoring ~ 1st—3rd years after					Pre-monitoring and final-monitoring (3rd year)				
Calving Interval	*n*	Mean	SEM	*p*-value	Calving Interval	*n*	Mean	SEM	*p*-value
Pre-ZEN monitoring (2019)	18	471.7	33.1	0.007*	Pre-ZEN monitoring (2019)	18	471.7	33.1	0.005*
Post-ZEN monitoring (2020, 2021, 2022)	58	388.2	10.3		Post-ZEN monitoring (2022)	20	368.3	10	

n: number of births (calving); *: significant difference, *p*-value < 0.05

DISCUSSION

The primary goal of this feed hygiene monitoring is to raise awareness of the adverse effects of mycotoxins on farmers. Acute exposure to excessive concentrations of mycotoxins in cattle frequently results in well-defined clinical signs such as decreased feed intake or diarrhea. Although ZEN has been shown in laboratory animal studies to cause oxidative stress and cell death [Abbes *et al.,* 2020; Zang *et al.,* 2020], clinical symptoms of hyperestrogenism in ruminating cows are uncommon [Weaver *et al.,* 1986; Fink-Gremmels, 2008]; in our previously reported ZEN-contaminated herds, we were unable to identify the characteristic clinical signs of ZEN [Hasunuma *et al.,* 2012], but only diarrhea. Sub-chronic and chronic low-dose exposure has been less thoroughly studied; however, it is responsible for decreased performance and potentially the reproductive efficacy of the herd [Guerrero-Netro *et al.,* 2015]. We used the calving interval as the key indicator for long-term monitoring (two-year surveys) of urinary ZEN to confirm the results on cattle reproductive performance with parameters that might be affected by reproduction. The calving interval for this herd gradually decreased from before monitoring (471.7 days), demonstrating a significant change from the current calving interval (368 days). In addition, the annual herd calving rate increased from 18 to 20 calves. Subsequent long-term urinary ZEN monitoring showed that the concentration of urinary ZEN was low from autumn, when the new rice straw was introduced until early spring of the following year, and then increased in the hot and humid spring and summer. In our previous field study, mycotoxin adsorbent (MA) or oligosaccharide (DFA III) supplementation of ZEN-contaminated feed reduced ZEN absorption in the gut of cattle [Takagi *et al.,* 2011; Fushimi *et al.,* 2014]. Therefore, in spring and summer, the administration of MA and DFA III could be an appropriate method to prevent ZEN absorption under low-level chronic ZEN contamination. As pointed out in our previous report [Widodo *et al.,* 2022], urinary ZEN monitoring system is practically useful for herd management and reproductive efficacy in breeding cattle herds.

In our previous study, based on the results of 1 year of monitoring from July 2020 to June 2021, we obtained the tendencies of the relationship between urinary ZEN and AMH concentrations, and those between urinary ZEN and SAA concentrations [Widodo *et al.,* 2022]. In the present study, although no significant relationship was obtained based only on the 2nd-year data, as shown in **Tables 1 and 2**, significant co-relationships among the urinary ZEN, AMH, and SAA concentrations in the breeding herd based on the whole 2 years of data were obtained, as shown in **Tables 3 and 4**, and we could show the relevance

of a clear formula regarding the relationship. The results obtained based on 2 years of data revealed the following: (1) there was a negative correlation between urinary ZEN concentration 2 months earlier and AMH concentration in the current month, (2) there was a negative correlation between the ZEN concentration 1 month ago and that of the current month, (3) the AMH concentration 1 month ago and that in the current month have a negative correlation, and (4) there is a negative correlation between the SAA concentration one month ago and that of the current month. Summarily, it is statistically clear that the instructions and guidance given to farmers based on the results of ZEN-AMH-SAA concentrations being monitored are correctly reflected in the results of the current month. Therefore, it was strongly speculated that feeding hygiene guidance based on our monthly monitoring data for two years led to the above-mentioned improvement in the fertility of the breeding cattle herd. However, new findings regarding AMH concentrations in aging JB cows were obtained from our monitoring. It has been reported that multiparous cows have higher AMH concentrations than primiparous cows throughout the postpartum period, suggesting that plasma AMH concentrations are higher in older cows throughout the postpartum period [Koizumi *et al.,* 2017]. The AMH concentrations of fixed cows measured monthly for 2 years (**Figure 2**) revealed that although the AMH concentration fluctuated (increased or decreased) due to the urinary ZEN concentration, the cow AMH concentration in this herd statistically decreased with aging during the 2-year monitoring period. To the best of our knowledge, this is the first report on AMH decline with age in JB breeding cows, which may indicate a decline in the number of antral follicles in aging cows. Further research using data from additional cows is required to clarify this phenomenon.

These findings indicate that the urinary ZEN monitoring system may be a valuable practical tool for screening and detecting herd contamination in the field, and acute and/or chronic ZEN contamination in dietary feeds may affect herd productivity and the fertility of breeding cows.

SUPPLEMENTARY MATERIAL

Table S1. The raw data of ZEN, AMH, and SAA measurement results

Date	Cow numbers (ear tag number)																	
	1 (0842)			2 (2291)			3 (2646)			4 (2899)			5 (4622)			6 (6074)		
	ZEN	AMH	SAA	ZEN	AMH	SAA	ZEN	AMH	SAA	ZEN	AMH	SAA	ZEN	AMH	SAA	ZEN	AMH	SAA
2020/Jul	2257.7	1230	5.1	1382.5	1357	3.3	4366	1290	1.8	2445.4	1241	2.1	1353.7	2490	2.5	-	-	-
2020/Aug	9021.1	1247	2.9	5802.3	674	23.4	9662.3	1441	2.1	-	-	-	5222.3	2099	2.2	-	-	-
2020/Sept	335.5	1506	2.2	324.9	870	3.6	668.1	1445	1.9	915.8	937	5.7	576.1	2032	2.3	-	-	-
2020/Oct	1585.1	1219	2.1	800.8	1093	18.1	946.2	1294	2.3	925.4	1252	4.5	1236.3	1925	2.5	-	-	-
2020/Nov	586.5	954	3.1	618.2	1853	3.1	609.8	1331	5	803.4	1481	2.5	471.4	1465	3.2	-	-	-
2020/Dec	1146.9	1338	4.7	467.2	2429	3.5	590.5	1548	2.7	1616.4	1696	2.3	380.3	1318	2.6	-	-	-
2021/Jan	699.4	1759	6.1	520.3	2639	26.8	884.2	1513	2.5	936.5	1890	2.4	471.5	1303	2.9	-	-	-
2021/Feb	1284.8	1979	5.5	886.8	1985	3.3	730.9	1563	2.2	1678.9	1918	2.3	631.3	744	2.9	-	-	-
2021/Mar	712.8	1864	5.5	618.2	1950	19.7	924.6	1073	4.9	769	2140	2.7	520	1351	3.1	-	-	-
2021/Apr	3866.8	1808	3.4	1604.4	1377	3.8	3784	1185	4.7	9710.6	2099	2.3	1460.1	1300	2.8	-	-	-
2021/May	1178.7	1931	2.8	1899.8	1221	2.7	2500.3	1399	1.1	3589.3	1910	1.9	849.1	2061	14.5	-	-	-
2021/Jun	1814	1552	3.0	763.6	964	7.4	2012.2	1564	1.6	1785.8	1600	1.8	782.4	1505	2	-	-	-
2021/Jul	1534.5	1827	1.7	2397.7	886	3.1	2945.7	2174	1.2	3237.6	1466	2.1	1650.8	1902	1.3	-	-	-
2021/Aug	1370.8	1635	1.2	1434.8	933	17.4	2901	2015	8.1	2738.7	1097	4.1	1207.2	1599	1	-	-	-
2021/Sept	557.1	663	20.3	831	938	2.7	2778.2	1727	1.5	4948	1105	13.3	2752.4	984	7.5	-	-	-
2021/Oct	313.9	921	3.2	641.3	1970	5.0	732.4	1911	1.7	860.8	1253	35.1	565.9	1271	1.7	-	-	-
2021/Nov	910.4	1134	5.7	576.4	2262	3.1	502.9	1232	2.3	957.8	734	6.7	425	511	1.7	-	-	-
2021/Dec	518.8	1211	4.5	830.9	2140	0.5	722.9	1456	0.8	1132.9	1499	17	604.8	777	2.9	-	-	-
2022/Jan	711.2	1767	0.2	361.9	2157	0.1	491.4	1248	0	545.9	1685	0	558.7	713	4.6	-	-	-
2022/Feb	2410.4	1731	6.9	-	1504	-	1224.6	969	3.5	1334.9	1443	2.1	661.4	816	3.3	709.2	285	4.5
2022/Mar	697.1	1851	0.6	889.5	1725	0.6	848.6	709	7	-	-	-	266.8	1332	0.4	509.6	280	0.5
2022/Apr	627.2	2059	3.9	746.4	1451	5.2	1312.8	923	6.3	-	-	-	566.9	1423	2.8	245.1	407	8.2
2022/May	514.7	1622	2.3	904	1082	1.1	815.3	1003	4.3	-	-	-	383.7	1964	0.5	684.4	635	1
2022/Jun	995.8	1374	1.0	453.5	982	2.2	1166.2	1625	0.7	-	-	-	409.8	1766	10.9	610.2	528	1.3
2022/Jul	333.3	1680	0.9	-	569	23	461	2075	0.9	-	-	-	438.4	2196	1.3	415.9	671	1.6

ZEN (pg/mg Cre); AMH (pg/mL); SAA (mg/L).
Cow 6 is a replacement cow from the same herd after Cow 4 was culled; Sampling could not be performed for Cow 4 in August 2020 because she was about to calve at the time of sampling; ZEN and SAA Cow 2 were not obtained in February 2022, nor was ZEN in July 2022.

Chapter 3

Exposure of cattle breeding herds to naturally co-contaminated zearalenone and deoxynivalenol: the relevance of a urinary mycotoxin monitoring system for herd health and food safety

ABSTRACT

The widespread presence of *Fusarium* mycotoxins in animal feed is a global issue, not only for the health of livestock but also for ensure the safety of food as an end product. High concentrations of zearalenone (ZEN) and deoxynivalenol (DON) have been detected in the diets of Japanese Black (JB) and Holstein Friesian (HF) breeding herds. Consequently, we monitored serum biochemical parameters over a long time in both herds, focusing on anti-Müllerian hormone (AMH) levels and acute-phase inflammation. Additionally, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and progesterone levels were measured in the HF herd. The JB herd, a ZEN-dominant model with low DON contamination, demonstrated ZEN levels that exceeded the Japanese limit in the purchased total mixed rations (TMR). Conversely, the HF herd, which primary consumes DON-dominant feed with low ZEN contamination, had high DON levels in the dent corn silage. Specifically, the JB herd's TMR contained 1.79 mg/kg ZEN and 0.58 mg/kg DON, whereas the HF herd's silage had 15.3 mg/kg DON (dried sample) and 0.1 mg/kg ZEN. Enzyme-linked immunoassay were used to measure urinary ZEN-DON levels following confirmation through liquid chromatography-tandem mass spectrometry. Urinary ZEN-DON levels measured were significantly correlated ($p < 0.05$, $r > 0.6$) in both herds. In the HF herd, AMH levels increased ($p = 0.01$) and serum amyloid A (SAA) levels decreased ($p = 0.02$) when contaminated and at the end of the monitoring period. Additionally, urinary ZEN and DON levels were significantly correlated with SAA levels (ZEN: $p = 0.00$, $r = 0.46$; DON: $p = 0.03$, $r = 0.33$), with an increase in ZEN and DON levels resulting in higher SAA levels. The JB herd showed no significant differences. Additionally, in the HF herd, 8-OHdG/Cre levels increased significantly during major contamination periods ($p < 0.05$). Clinical data from the HF herd indicated an increase in mastitis cases and treatment rates during periods of major contamination. Abortion rates in the HF herd decreased from 22.9% (before monitoring) to 8.9% (during the high contamination period) and finally to 1% (at the end of the monitoring period), with corresponding increases in progesterone levels. ZEN-DON contamination adversely affects breeding cattle's productivity, reproductive performance, and health. Therefore, monitoring urinary ZEN-DON is valuable for detecting contaminants and ensuring the safety of food products.

INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi that contaminate cattle feed at various stages, including the field growth, harvest, transportation, feed manufacturing, and storage [Alonso *et al.,* 2013; Liu *et al.,* 2020; Vandicke *et al.,* 2021]. The widespread presence of *Fusarium* mycotoxins in animal feed is a global issue for the health of livestock and for the safety of food as an end product. We monitored beef and dairy cattle herds to examine the dynamics of mycotoxin contamination at meat and milk production sites, which are major sources of human food. Furthermore, food safety depends not only on mycotoxin contamination of livestock products but also on the health of cattle at the source of contamination. Previously, researchers have studied its toxicity, its effect on organisms, and methods to control these compounds. Zearalenone (ZEN) is an estrogenic mycotoxin that interferes with cattle reproductive functions and is frequently found in grains and feed [Gruber-Dorninger *et al.,* 2019; Eskola *et al.,* 2019; Thapa *et al.,* 2021]. We previously developed and validated a urinary ZEN monitoring system for cattle using an enzyme-linked immunoassay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). This study aimed to investigate production sites and determine the impact of mycotoxin contamination on cattle health and fertility [Takagi *et al.,* 2011; Hasunuma *et al.,* 2012; Widodo *et al.,* 2022]. In addition, we evaluated the efficacy of ZEN adsorbents added to feed in reducing mycotoxin absorption using a urine ZEN monitoring system [Takagi *et al.,* 2011]. Our recent study [Widodo *et al.,* 2023] explored the effects of ZEN contamination on two biomarkers: anti-Müllerian hormone (AMH), a potential marker of fertility, ovulatory hyper-response, ovarian dysfunction, and herd longevity [Mosa *et al.,* 2017, 2019; Umer *et al.,* 2019; Ramesha *et al.,* 2022], and serum amyloid A (SAA), an acute-phase protein associated with inflammation [Eckersall *et al.,* 2010; Chan *et al.,* 2010; Bazzano *et al.,* 2022; Shinya *et al.,* 2022].

Several types of fungi produce multiple toxins. The two main mycotoxins produced by *Fusarium* species are ZEN and deoxynivalenol (DON). DON is cytotoxic and immunotoxic and can impair protein synthesis. Susceptibility to DON varies among animals, with ruminants being fairly resistant and pigs being particularly vulnerable [Pestka, 2007]. Co-contamination with ZEN and DON is common, occurring in approximately 23.8% of 400 feedstuff samples tested and 27% of cattle feed samples evaluated [Muñoz-Solano and González-Peñas, 2023]. Improved detection tools have enabled the identification of multiple

mycotoxins in feed, aiding the development of management strategies to minimize their adverse effects on cattle health and reproductive performance.

Exposure tests using in vitro cultured cells are important for evaluating toxicity at the genetic level [Guerrero-Netro *et al.,* 2015; Novak *et al.,* 2018; Fu *et al.,* 2022]. To elucidate the effects of mycotoxin contamination on cattle health and fertility, it is important to investigate naturally occurring cases. In our ongoing investigation of ZEN infiltration in cow herds, we confirmed ZEN and DON contamination in the feed of two breeding herds: Japanese Black (JB) and Holstein Friesian (HF). This study used these herds as models for ZEN- and DON-dominant contamination to evaluate its effects on health and fertility. We verified the efficacy of the DON measurement system in urine samples using a commercially available ELISA kit. Additionally, we conducted blood biochemical tests, measuring levels of progesterone (P4) and urinary 8-hydroxy-2'- deoxyguanosine (8-OHdG), a biomarker of DNA oxidative stress [Giromini *et al.,* 2016; Olarotimi *et al.,* 2023]. Furthermore, we verified the relationship between changes in conception rate (CCR), abortion rate, disease incidence, and mycotoxin concentrations in urine and feed, based on the clinical records of both herds.

MATERIAL AND METHODS

Chemicals and Solvents

ZEN was purchased from MP Biomedicals (Heidelberg, Germany), and metabolites α-ZEL and β-ZEL were purchased from Sigma (St. Louis, MO, USA). Mycotoxin mixtures including DON and 3-Ace-DON were obtained from Romer Labs, Inc. (Newark, NJ, USA). Stock solutions of ZEN, α-ZEL, and β-ZEL (1 μg/mL in methanol) were stored under light protection at 4 ˚C. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan) and Sigma-Aldrich, respectively. β-Glucuronidase/arylsulfatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan), and Tris was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Oasis HLB Vac Cartridge (6 cc, 2 mg sorbent, 30 μm) was purchased from Waters Co. (Milford, MA, USA).

Identification of Mycotoxin Co-Contaminated Herds as ZEN-Dominant and DON-Dominant Models

- JB Breeding Herd as ZEN-Dominant Model

In July 2022, at the request of the managing veterinarian, two JB breeding herds (A and B) in a neighborhood in the Kyushu area of Japan were subjected to urinary ZEN monitoring. The sudden decrease in monthly pregnancy test results in Herd A prompted a request for testing. The urinary ZEN concentration in herd A was high based on our past reported values [Widodo *et al.,* 2022]. As ZEN contamination was strongly suggested in herd A, we informed the managing veterinarian of the test results, recommended the measurement of mycotoxin concentrations in the feed, and requested the collection of urine and blood samples in August. Subsequent measurements of mycotoxin concentrations in the total mixed rations (TMR) feed diet of herd A confirmed that ZEN contamination exceeded the Japanese standard value (1 mg/kg). However, urinary ZEN monitoring in herd B demonstrated levels of suspected contamination below 1 mg/kg in the feed, as per our previous report [Widodo *et al.,* 2023]. Based on the mycotoxin test results obtained from herd A feed samples, we recommended that the potentially contaminated TMR be discontinued and that the amount of TMR that is discontinued be replaced with other non-contaminated roughages such as rice straw.

- *HF Breeding Herd as DON-Dominant Model*

In July 2022, the managing veterinarian of a dairy farm in the Honshu area of Japan suspected mycotoxin contamination of the cattle feed. This suspicion was based on the observed decline in conception rates, an increase in abortion rates, and an upward trend in mastitis cases. We identified a significant amount of DON in the feed sample, which led the company to initiate discussions on future strategies. Based on the mycotoxin test results obtained from the feed samples, we recommended reducing the amount of potentially contaminated corn silage by half and replacing it with uncontaminated roughage. Blood and urine samples were collected immediately. Our proposal includes both sample collection and continuous monitoring of mycotoxin concentrations using urine and blood samples.

Based on the background described above, these two herds will be used as ZEN (JB herd) and DON (HF herd) contaminated model herds, and after subsequent countermeasures have been taken, urine and blood sampling will be continued, and clinical records will be collected as much as possible.

Dietary Components, Experimental Design, and Sample Collection

The daily feed ratios for each breeding herd are listed in **Table 1**. In the JB herd, TMR and oat hay were commercially available, whereas WCS and Italian ryegrass silage were homegrown. Concentrate was provided only to cows two months before delivery. Farm HF used two lots of dent corn silage to reduce mycotoxin levels. The contaminated feed was supplemented with fresh uncontaminated feed. The study included two herds with naturally occurring mycotoxin co-contamination, each receiving the same feeding regimen. The JB herd in Kyushu consisted of 29 cows (average age, 3 years and 11 months) with five sample sessions from July to December 2022. The HF herd in Honshu consisted of 30 cows (average age, 3 years and 2 months) with six sampling sessions from August 2022 to May 2024. Additional monitoring will be performed in May 2024. **Figure 1** shows the monthly temperature data and schematic of the experimental design. Urine, blood, and roughage samples were collected from the JB (n = 3 urine, n = 9 blood) and HF (n = 10 urine, n = 30 blood) herds following procedures [Takagi *et al.,* 2011]. The samples were promptly cooled, transported to the laboratory, and stored at −30 ˚C after centrifugation. Roughage samples were collected to measure the mycotoxin concentrations. Urine samples from at least two cows were considered sufficient to assess the feed contamination levels [Widodo *et al.,* 2022].

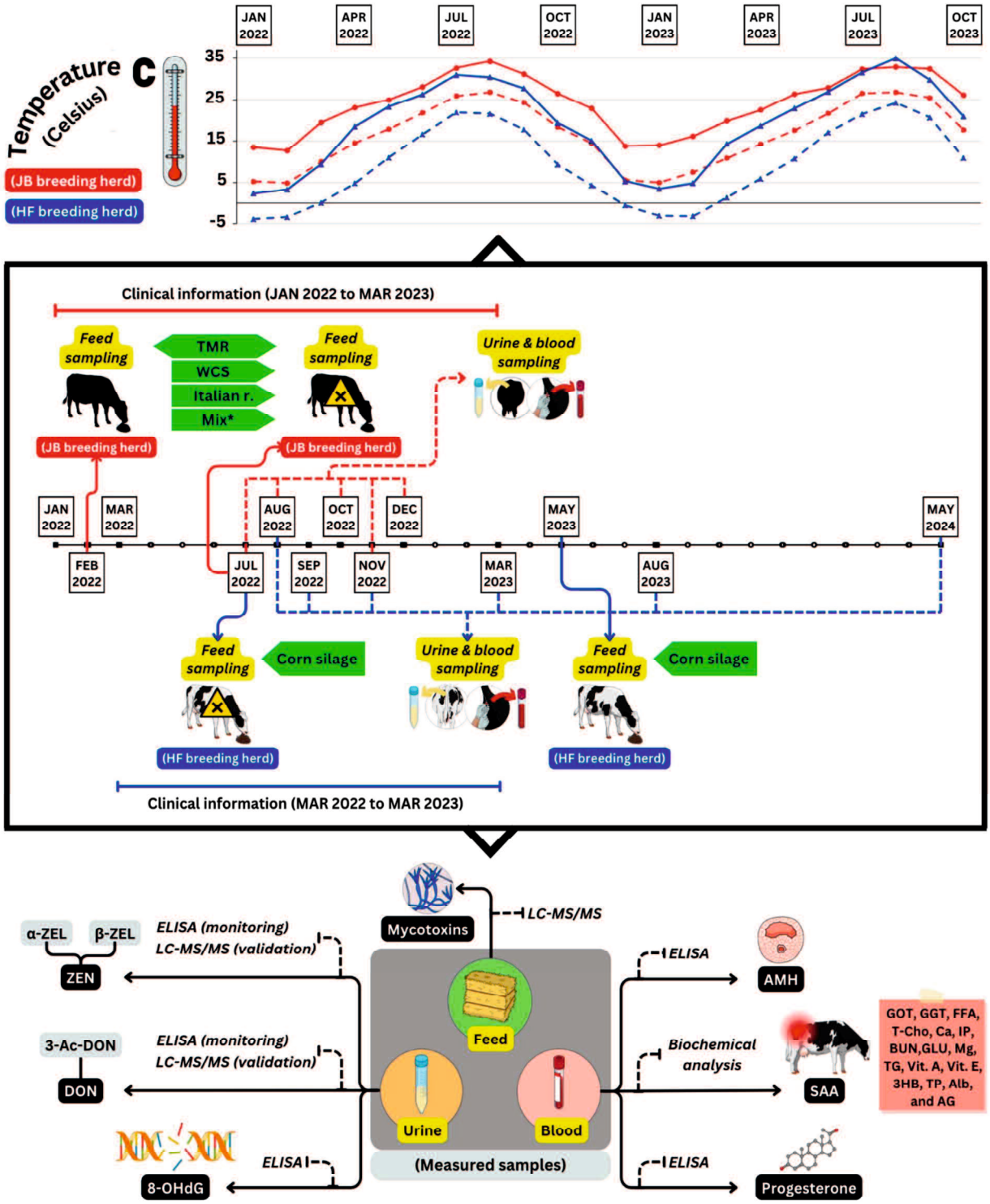

Figure 1. An illustration of the experimental design. The design includes monthly changes of highest and lowest temperatures in degrees Celsius (°C) for the JB and HF breeding herds, as well as comprehensive information on sampling time and the biomarkers analyzed in feed, blood, and urine samples. A harmful mark indicates the first time mycotoxin contamination was detected in feed on both herds (July 2022); TMR: total mixed ratio; WCS: whole crop silage; Italian r.: Italian ryegrass; Mix*: mixture of TMR, WCS, and Italian ryegrass; ZEN: zearalenone; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol; DON: deoxynivalenol; 3-Ac-DON: 3-acetyldeoxynivalenol; AMH: anti-Müllerian hormone; SAA: serum amyloid A; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

Table 1. Daily feed ration in the JB and HF breeding herd

Breeding Herd	Forage Feds	Formula Feeds
Japanese Black (JB)	WCS (rice) [1.5 kg]	TMR [4 kg]
	Italian ryegrass-silage [1.5 kg]	Concentrate * [1.5–2 kg]
	Oats-hay [1 kg]	Calcium-mineral mix [0.04 kg]
Holstein Friesian (HF)	Timothy grass [0.2 kg]	Okara (soybean curd) [7.5 kg]
	Alfalfa grass [1 kg]	Compound feed [7.5 kg]
	Oats-hay [2 kg]	Steam pressed yellow corn [2 kg]
	WCS [7.5 kg]	Wheat bran [1 kg]
	Dent corn-silage (24.5% DM) [8 kg]	Rice bran [2.5 kg]
	Dent corn-silage (27.6% DM) [4.6 kg]	Calcium-mineral mix [0.16 kg]

WCS, whole crop silage; TMR, total mixed ration; Concentrate *: two months before delivery.

Analytical Methods of Mycotoxins in Feed Samples

In this study, private laboratories conducted measurements of mycotoxin concentrations in feed for each herd. Parallel measurements of mycotoxins (ZEN, DON, aflatoxin B1, ochratoxin A, patulin, fumonisin B1, nivalenol, and sterigmatocystin) were performed using liquid chromatography-tandem mass spectrometry. These measurements followed the multicomponent analysis of mycotoxins as specified in the feed analysis standards of the Food and Agricultural Products Inspection Center, Japan. The screening results for mycotoxin concentrations in feed from the two herds revealed the presence of ZEN and DON at concentrations exceeding the Japanese standard. However, the study excluded other mycotoxin concentrations due to their too low or undetectable levels.

Analytical Methods of ZEN in Urine Samples

Urinary creatinine concentrations were determined using a commercial kit (Sikarikit-S CRE; Kanto Chemical, Tokyo, Japan) and measured using a 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan). All urine concentrations were expressed as a ratio of creatinine (pg/mg creatinine). ZEN concentrations in the urine were analyzed using an ELISA kit (RIDASCREEN Zearalenon; R-Biopharm AG, Garmstadt, Germany). A urine sample (0.1 mL: 5-fold dilution of the kit) was mixed into 3 mL of 50 mM sodium acetate buffer (pH 4.8) and incubated at 37 ˚C for 15 h with 10 µL of β-glucuronidase/arylsulfatase solution. The samples were then placed on a C18 solid-phase extraction (SPE) column (Strata; Phenomenex, Torrance, CA, USA) preconditioned with 3 mL of methanol and 2 mL

of 20 mM Tris buffer (pH 8.5)/methanol (80:20). The SPE column was washed with 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20) and 3 mL of 40% methanol and then centrifuged for 10 min at 500× *g* to dry. Analytes were eluted gently (15 drops/min) with 1 mL of methanol (80%). From the 1 mL of 80% methanol solution, 0.8 mL was refrigerated for LC-MS/MS measurements, and 0.2 mL was used for ELISA assays. A centrifugal evaporator was used to dry the eluate at 60 ˚C. To perform the ELISA, the dried residue was redissolved in 50 μL of methanol, 450 μL of sample dilution buffer was added, and the solution was thoroughly mixed. An aliquot of 50 μL was used for the ELISA assay. RIDA SOFT Win (R-Biopharm) was used to determine absorbance at 450 nm using a microplate spectrophotometer. The cross-reactivity rates using this particular ELISA kit for α-ZEL, β-ZEL, and Zeranol were 41.6, 13.8%, and 27.7%, respectively, with a mean recovery rate of 84% ± 14%.

Samples with high ZEN levels were further analyzed using LC-MS/MS to confirm ELISA results and measure ZEN metabolites, α-ZEL and β-ZEL [Takagi *et al.,* 2011; Widodo *et al.,* 2022]. Each urine sample (0.5 mL) was mixed with 3 mL of 50 mM ammonium acetate buffer (pH 4.8) and 8 μL of glucuronidase/arylsulfatase solution and incubated for 12 h at 37 ˚C. The solution was loaded onto a C18 SPE column, which was preconditioned with 3 mL 100% methanol and 2 mL Tris buffer, followed by the addition of 2 mL Tris buffer and 3 mL 40% methanol. After washing the SPE column with approximately 1 mL 80% methanol, the volume of the eluted solution was adjusted to 1 mL. Then, 5 μL of the reconstituted solution was injected into the LC-MS/MS system (AB Sciex Qtrap 4500 system, Applied Biosystems, Foster City, CA, USA) with the electrospray ionization (ESI) source in the negative mode. The detection limits for ZEN, α-ZEL, and β-ZEL in urine samples were all 0.005 ng/mL, while the mean recovery rates for ZEN, α-ZEL, and β-ZEL were 90%, 109%, and 90%, respectively. Chromatographic separation for ZEN and the metabolites was performed on an InertSustain C18 column (2.1 i.d. × 100 mm, 2 μm; GL Sciences, Tokyo, Japan) at 40 ˚C using the mobile phase consisting of solvent A (water) and solvent B (methanol). An optimized gradient elution with a flow rate of 200 mL was 50% to 100% B from 0 to 5 min and then held at 100% B for 9 min. The operation of Qtrap was optimized as follows: curtain gas (CUR), 20 psi; ion source (IS) gas 1 and gas 2, 60 and 30 psi; ionspray voltage (IS), −4500 V; temperature (TEM), 500 ˚C; declustering potential (DP), −125 V for ZEN, −120 V for ZEN metabolites; entrance potential (EP), −10 V; collision energy (CE), −32 V for ZEN, −30 V for metabolites; and collision exit potential

(CXP), −9 V for ZEN, −11 V for metabolites. Mass transitions were 317.0 to 175.0 for ZEN and 319.1 to 275.0 for ZEN metabolites.

Analytical Methods of DON in Urine Samples

- Preliminary Trial for Urinary DON Measurement by ELISA

The DON concentration in the urine was determined using a commercially available ELISA kit (RIDASCREEN deoxynivalenol; R-Biopharm AG, Darmstadt, Germany). Although the assay kit instructions did not mentioned which urine samples were suitable for testing, a preliminary test was conducted to confirm the reliability of the assay for bovine urine samples. The test used mycotoxin mixture solution 4 (Kanto Chemical Co., Inc.) and Biopure Mix 4 (Romer Lab), both containing 10 mg/L 3-Ace-DON, Deoxynivalenol, Nivalenol (NIV), Fusarenon X, HT-2 Toxin, T-2 Toxin, Diacetoxyscirpenol, and Zearalenone. These solutions served as the standard solutions for the recovery test. Briefly, a mixture of the urine samples from 3 different HF cows supplemented with 100 μg/L (ppb), 10 μg/L, 1 μg/L, and 0 μg/L of DON (also containing 3-Ace-DON) was used for the recovery tests. Additionally, recovery rates of distilled water supplemented with 100 μg/L (ppb), 10 μg/L, 1 μg/L, and 0 μg/L of DON were also examined. The recovery test was performed twice. The mean recovery rates of DON were ND in the case of supplementation with 100 μg/L (ppb), 542% in the case of supplementation with 10 μg/L, and 585% in the case of supplementation with 1 μg/L. Based on the cross-reactivity data provided by the ELISA kit used in this study, the antibodies showed 100% reactivity with DON, over 100% reactivity with 3-Ace-DON, 19% reactivity with 15-Ace-DON, 4% reactivity with NIV, and less than 1% reactivity with T-2 Toxin. However, different cross-reactivity values were obtained for commercially available DON-ELISA kit antibodies, indicating that the cross-reactivity of 3-Ace-DON in the ELISA kit used in this study was 520 ± 110% and 15-Ace-DON was 3 ± 2% [Nguyen *et al.,* 2019]. The results of our preliminary study are consistent with those of a previous report [Nguyen *et al.,* 2019]. The ELISA kit proved to be valuable for detection and screening of "DON groups" in bovine urine samples. Subsequently, we established and validated this assay using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

- Preliminary Trial for Urinary DON Measurement by LC-MS/MS

For LC-MS/MS analysis, preliminary trials were conducted using direct extraction and pre-incubation with β-glucuronidase and arylsulfatase. The DON was extracted using a

commercial immunoaffinity column (DONPREP; R-Biopharm AG, Garmstadt, Germany). After placing a 2 mL urine sample through the column, 10 mL of distilled water was passed through at a rate of 5 mL/minute with air to remove the remaining liquid. The toxin was eluted from the column at 1 drop per second using 100% methanol (1.5 mL) and collected in a glass container. The HLB column was loaded with 0.5 mL of methanol solution and 4.5 mL of Milli-Q. After loading, the column was removed by liquid centrifugation (2,500 rpm for 10 min). The target mycotoxins were eluted with 1 mL of methanol/water (4:6, *v*/*v*) and 1 mL of methanol. A glass vial container was used for each elution as described below.

Preliminary trials for extraction methods were conducted to confirm the appropriate quantification of DON and 3-Ace-DON in bovine urine. LC-MS/MS, direct extraction, and pre-incubation with β-glucuronidase/arylsulfatase were conducted. Subsequently, we performed recovery tests. DON recovery rates were 169% with 100 μg/L (ppb), 167% with 10 μg/L, and 105% with 1 μg/L, while 3-Ace-DON recovery rates were 156% with 100 μg/L (ppb), 236% with 10 μg/L, and 462% with 1 μg/L. DON recovery rates in urine samples from 1 HF cow supplemented with 100 μg/L (ppb), 10 μg/L, 1 μg/L, and 0 μg/L DON with or without β-glucuronidase/arylsulfatase were 82% and 67% with 100 μg/L (ppb), 88% and 93% with 10 μg/L, and 374% and 87% with 1 μg/L. Sample 1 (1.5 ppb and 1.2 ppb) and sample 2 (0.45 and 0.72 ppb) had urinary DON concentrations with or without β-glucuronidase or arylsulfatase supplementation. In conclusion, the LC-MS/MS system for assessing urine DON concentration is a suitable screening approach, and adding β-glucuronidase or arylsulfatase during extraction does not significantly impact the results.

- DON Measurement by LC-MS/MS

The final LC-MS/MS method used an InertSustain C18 column (2.1 i.d., 100 mm, 2 m) in the negative mode to target DON, 3-Ace-DON, and NIV for Qtrap 4500 systems. The detection limit was 0.025 ng/mL in urine when the injection volume was 5 μm. We measured DON, 3-Ace-Don, and NIV under gradient conditions, using a 200-L flow rate. We measured 2% solvent B (methanol) from 0 to 2 min, 2% to 98% B from 2 to 8 min, and maintained 98% B for 12 min. We optimized Qtrap's operation as follows: The operating pressure was set at 20 psi for CUR, 80 psi for IS gas 1 and 2, 80 psi for gas 2, and 400 ˚C for TEM. Mass transitions were 295.0 to 265.0 for DON, 337.0 to 307.0 for 3-Ace-DON, and 310.9 to 281.0 for NIV.

Analytical Method of Biochemical Parameters in Serum Samples and Urinary Levels of 8-OHdG

Blood samples (10 mL) from the jugular vein were analyzed for various biochemical parameters using a LaboSpect 7080 autoanalyzer (Hitachi, Tokyo, Japan). Serum vitamin A and E levels were measured via HPLC (Shimadzu, Kyoto, Japan) to assess vitamin depletion. The tests monitored hepatic, renal, and nutritional status, as well as mineral intake. The AMH concentration was measured using a bovine ELISA kit (AnshLabs, Webster, TX, USA) with a detection limit of 11 pg/mL [Fushimi *et al.,* 2019]. SAA concentrations were determined using an automated biochemical analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) with a specific reagent (VET-SAA 'Eiken'; Eiken Chemical Co., Ltd., Tokyo, Japan). Serum P4 concentrations were measured via enzyme immunoassay (COSMO FKA302-E, Tokyo, Japan) after diethyl ether extraction, with a detection limit of 0.05 ng/mL [Ono *et al.,* 2018].

To evaluate oxidative DNA damage, urinary 8-OHdG levels were measured using an ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Japan) with a detection limit of 0.5 ng/mL [Kantha *et al.,* 1996; Fan *et al.,* 2000]. Diluted urine with distilled water (50 µL: 3 times in HF herd and 20 times in non-contaminated control JB herd) was used for the assay. The absorbance of the samples and standards was measured at 450 nm using a microplate photometer (Multiskan FC; Thermo Fisher Scientific, Tokyo, Japan). 8-OHdG concentrations in the samples were interpolated from a standard curve using log transformation.

Data Management and Statistical Analysis

Correlation analysis quantifies the relationships between biomarkers with the data structure modified using medians to maintain consistent observations [Lai *et al.,* 2019]. The Kolmogorov–Smirnov test was used to determine the data distribution. For normally distributed data ($p > 0.05$), Pearson's correlation was used, and for non-normally distributed data ($p < 0.05$), Spearman's correlation was applied. p-values < 0.05, 0.05–0.10, and >0.1 indicated significant, tendency, and non-significant results, respectively. Correlation coefficients (r) were categorized as: 0.00–0.19 (very weak), 0.20–0.39 (weak), 0.40–0.59 (moderate), 0.60–0.79 (strong), and 0.80–1.00 (very strong). For ANOVA, normality (Kolmogorov–Smirnov test) and homogeneity (Levene test) were assessed. Post hoc tests included Duncan's test (homogeneous data) and the Games–Howell test (non-homogeneous data). For non-parametric data, the Kruskal–Wallis test was used, followed by the Mann–

Whitney U test for significant results. All analyses were performed using the IBM SPSS Statistics version 25.

RESULTS

Co-Contamination Status in Cattle Breeding Herds

Mycotoxin analysis of feed from JB and HF breeding herds (**Table 2**) revealed contamination with ZEN and DON. ZEN contamination was dominant in the JB herd, with the purchased total mixed rations (TMR) showing a peak level of 1.791 mg/kg, exceeding Japan's limit of 1 mg/kg. In contrast, the HF herd's feed was predominantly contaminated with DON, with corn silage containing 6.6 mg/kg, surpassing Japan's 4 mg/kg limit for calves older than 3 months.

Table 2. Results of the JB and HF breeding herd feed sample analyses

JB Breeding Herd				HF Breeding Herd			
Date	Feed Sample	ZEN (mg/kg)	DON (mg/kg)	Date	Feed Sample	ZEN (mg/kg)	DON (mg/kg)
2022/Feb	Purcased TMR	0.121	ND	2022/Jul	Dent corn silage[1]	0.1	3
2022/Jul	Purcased TMR	1.791	0.58	2022/Jul	Dent corn silage[2] (ori.)	0.062	6.6
2022/Jul	WCS (rice)	0.056	ND	2022/Jul	Dent corn silage[2] (dry)	0.145	15.3
2022/Jul	Italian ryegrass (produced in 2020)	0.079	ND	2023/May	Dent corn silage	ND	0.09
2022/Jul	Italian ryegrass (produced in 2021)	0.081	ND				
2022/Jul	Mix*	0.733	ND				

* Consists of purchased TMR, WCS rice straw, and Italian ryegrass (produced in 2021) in a feed combination (mix). [1,2] The same feed samples were sent to two feed analysis companies. Original sample (ori.), corn silage, had a moisture level of 57.1%. ZEN: zearalenone; DON: deoxynivalenol; ND: not detected.

An ELISA detected ZEN and DON in the urine of both herds. In the JB herd, the ZEN levels peaked in July 2022 and declined until December 2022 ($p = 0.06$), whereas DON levels showed no significant differences across the evaluated time period ($p = 0.55$). The HF herd exhibited a peak ZEN contamination in November 2022, with a significant decrease recorded thereafter ($p = 0.00$). DON levels in the HF herd were the highest in August and September 2022, followed by a significant decrease ($p = 0.00$). The ZEN levels in the HF herd were significantly higher from August–November 2022 than in March and August 2023 ($p < 0.05$, except for September 2022 vs. March 2023, $p = 0.06$).

LC-MS/MS validation of urine samples (**Table 3**) showed significant correlations with ELISA results for ZEN in both herds (JB: $p = 0.00$, $r = 0.94$; HF: $p = 0.00$, $r = 0.62$) and for DON in the HF herd ($p = 0.00$, $r = 0.75$). In the HF herd, significant differences in ZEN and DON levels were observed across sampling periods (both $p = 0.00$), whereas the JB herd showed a tendency toward significant differences in ZEN levels ($p = 0.055$). The

post hoc analysis results for the HF herd are presented in **Table S1** in Supplementary Materials. **Figure 2** illustrates the urinary co-contamination levels in both herds, as determined by ELISA and LC-MS/MS.

Table 3. The levels of urinary ZEN and DON in JB and HF herds determined through LC-MS/MS

Herd	Date	*n*	Urinary ZEN (Mean ±SEM)				Urinary DON (Mean ±SEM)		
			ZEN/Cre	α-ZEL/Cre	β-ZEL/Cre	Σ ZEN/Cre	3-Ac-DON/Cre	DON/Cre	Σ DON/Cre
JB	2022/Jul	3	3.66 ± 1.40	1.63 ± 0.68	3.32 ± 1.22	8.61 ± 3.28	-	-	-
	2022/Aug	2	0.12 ± 0.01	0.63 ± 0.07	0.92 ± 0.20	1.66 ± 0.26	-	-	-
	2022/Oct	2	0.12 ± 0.03	0.17 ± 0.02	0.32 ± 0.06	0.62 ± 0.08	-	-	-
	2022/Nov	2	0.13 ± 0.02	0.42 ± 0.16	0.05 ± 0.02	0.59 ± 0.16	-	-	-
	2022/Dec	2	0.04 ± 0.01	0.18 ± 0.05	ND	0.22 ± 0.06	-	-	-
HF	2022/Aug	10	1.57 ± 0.19	0.62 ± 0.06	2.01 ± 0.25	4.20 ± 0.48	0.14 ± 0.03	2.21 ± 0.06	2.35 ± 0.62
	2022/Sept	9	1.67 ± 0.31	0.65 ± 0.13	1.84 ± 0.35	4.16 ± 0.76	0.25 ± 0.04	3.51 ± 0.62	3.76 ± 0.63
	2022/Nov	9	1.03 ± 0.24	1.00 ± 0.19	0.24 ± 0.06	2.27 ± 0.47	0.10 ± 0.01	0.49 ± 0.10	0.59 ± 0.10
	2023/Mar	9	0.13 ± 0.02	0.07 ± 0.02	0.01 ± 0.00	0.22 ± 0.04	0.09 ± 0.02	0.27 ± 0.04	0.36 ± 0.05
	2023/Aug	9	2.69 ± 0.79	0.48 ± 0.04	0.09 ± 0.02	3.26 ± 0.85	0.10 ± 0.02	0.41 ± 0.06	0.51 ± 0.07

JB: Japanese Black; HF: Holstein Friesian; Cre: creatinine; ND: not detected; -: not evaluated; 3-Ac-DON: 3-acetyldeoxynivalenol; Σ: total value.

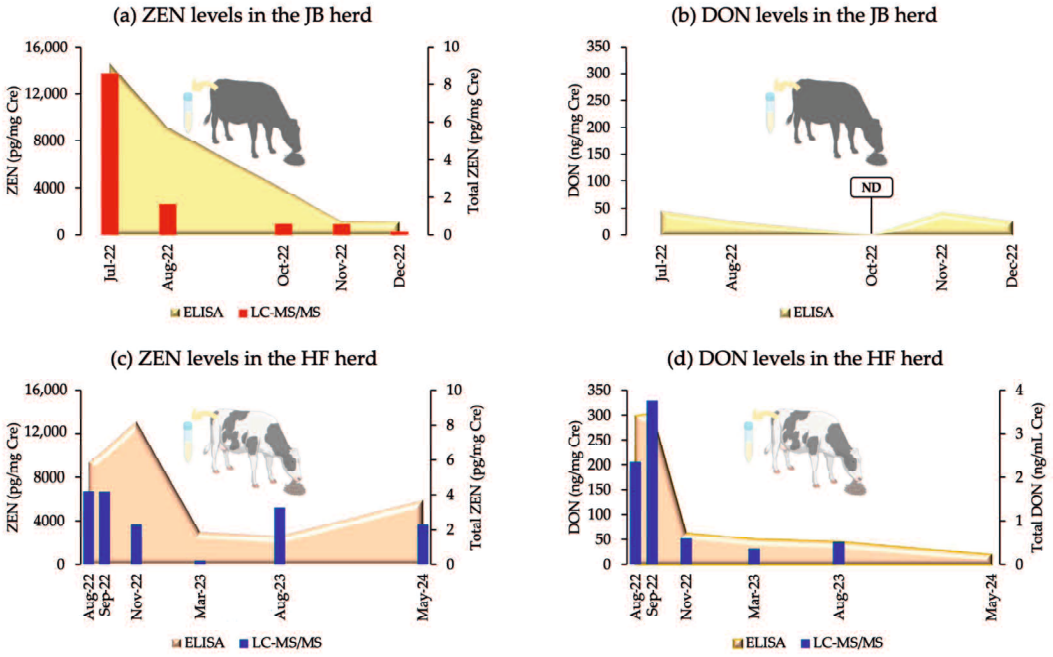

Figure 2. Urinary co-contamination levels in the JB and HF breeding herds, measured by an ELISA and LC-MS/MS. Cre: creatinine; ND: not detected; Total ZEN = ZEN/Cre + α-ZEL/Cre + β-ZEL/Cre; Total DON = DON/Cre + Ac-DON/Cre.

Biomarker Analysis

We examined multiple biomarkes including AMH, SAA, and blood biochemical parameters in both herds and 8-OHdG and P4 in the HF herd. In the JB herd, AMH levels

showed no significant differences across the periods ($p = 0.67$), whereas SAA levels showed a significant trend ($p = 0.05$). AMH levels (Mean ± SEM) from July to December 2022 were 945.89 ± 158.82, 880 ± 112.64, 1012 ± 227.05, 522.5 ± 89.86, and 888.8 ± 171.52, respectively, indicating a decline. SAA levels for the same months were 7.24 ± 2.36, 6.97 ± 2.73, 2.51 ± 1.21, 2.44 ± 0.48, and 10.52 ± 7.06.

In the HF herd, AMH levels (mean ± SEM) were 447.2 ± 53.91 in August 2022, decreasing to 412.2 ± 53.82 in September and 380.22 ± 54.99 in November, and then increasing to 450.70 ± 48.44 in March 2023 and peaking at 529.85 ± 65.97 in August 2023 ($p = 0.42$). SAA levels were 5.79 ± 0.81 in August 2022, peaking at 15.01 ± 5.67 in September, then decreasing to 12.03 ± 5.23 in November and 3.45 ± 0.92 in March 2023, with a small increase to 4.80 ± 1.72 in August 2023 ($p = 0.14$).

Comparing AMH and SAA levels from August 2022 to August 2023 showed significant differences (ps of 0.01 and 0.02, respectively), with AMH levels increasing and SAA levels decreasing. Both ZEN and DON were positively correlated with SAA in the HF herd (ZEN: $p = 0.00$, $r = 0.46$; DON: $p = 0.03$, $r = 0.33$), with increases in ZEN and DON levels corresponding to increases in SAA levels. **Figure 3** shows the AMH and SAA levels in both herds, as well as changes in SAA and urinary ZEN and DON levels.

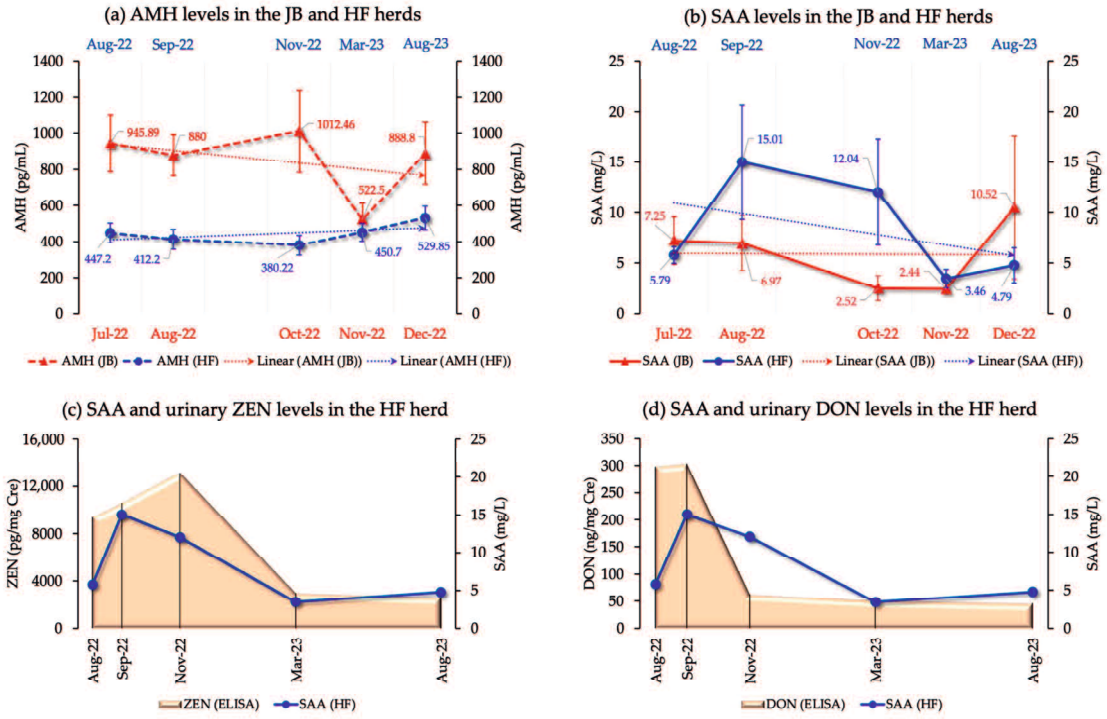

Figure 3. (a) AMH levels (trend lines, mean, and SEM) in both JB and HF breeding herds; (b) SAA levels (trend lines, mean, and SEM) in both JB and HF breeding herds; (c) Changes in both urinary ZEN and SAA levels in the HF breeding herd; (d) Changes in both urinary DON and SAA levels in the HF breeding herd.

Blood biochemical analyses were performed to measure the levels of GOT, GGT, FFA, T-Cho, BUN, Glu, Ca, IP, Mg, TG, Vit. A, Vit. E, 3HB, TP, Alb, and AG in the JB and HF breeding herds. In the JB herd, significant differences ($p < 0.05$) were observed across the breeding herds. In the JB herd, significant differences ($p < 0.05$) were observed across evaluated time period for almost all parameters, except GOT ($p = 0.06$), Ca ($p = 0.07$), GGT ($p = 0.45$), Mg ($p = 0.88$), and Alb ($p = 0.72$). In contrast, in the HF herd, significantences ($p < 0.05$) were found for all parameters except for Vit. E ($p = 0.24$) and TP ($p = 0.39$). Results of blood biochemical analyses (mean ± SEM) of the JB and HF breeding herd are presented in **Table S2**, as Supplementary Materials. The mean patterns of blood biochemical analyses for both herds are shown in **Figure 4**.

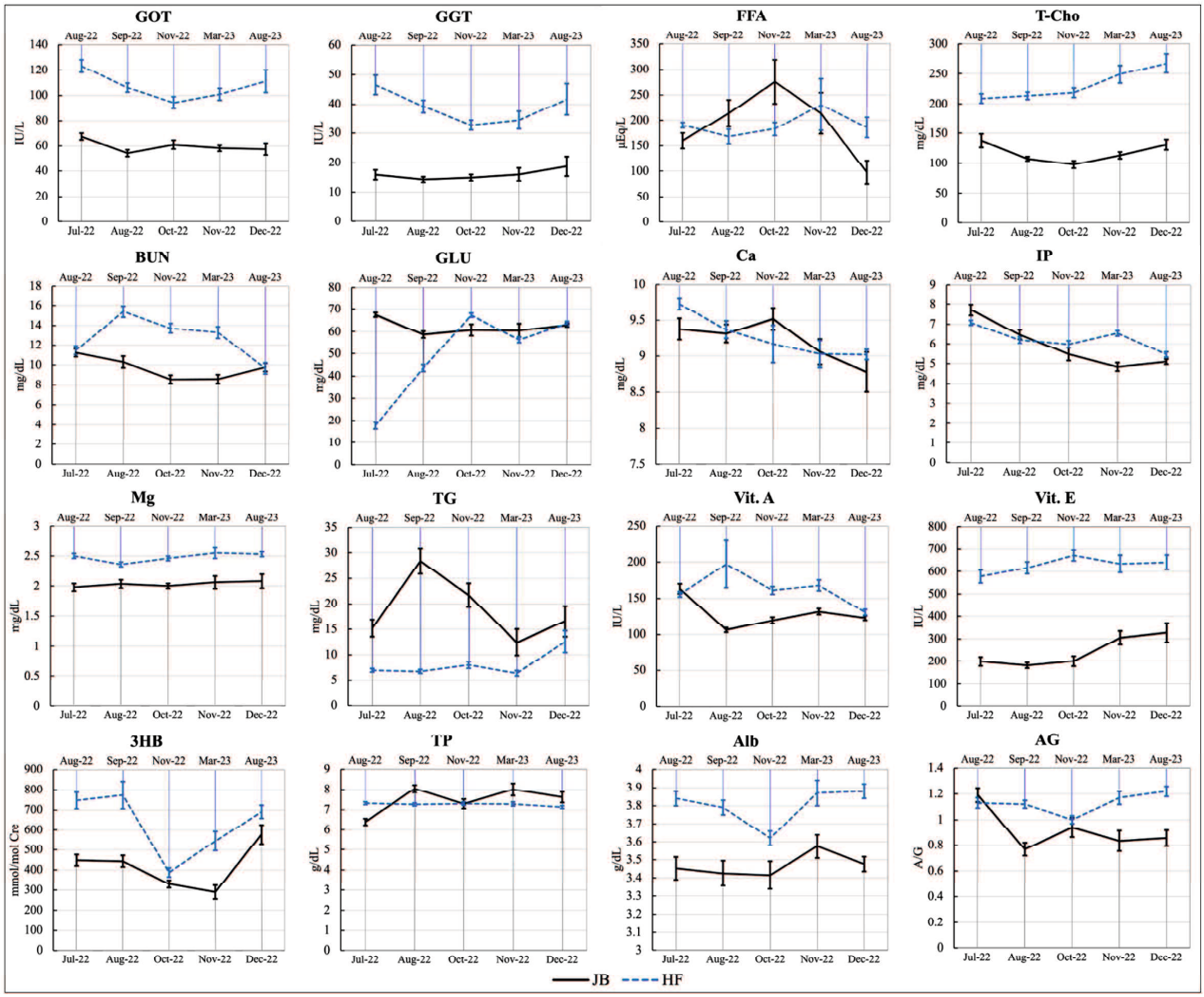

Figure 4. The patterns of blood biochemical analyses (mean ± SEM) of the examined cattle. GOT: glutamate-oxaloacetate transaminase; GGT: gamma-glutamyl transpeptidase; FFA: free fatty acids; T-Cho: total cholesterol; BUN: blood urea nitrogen; GLU: glucose; Ca: calcium; IP: inorganic phosphorus; Mg: magnesium; TG: triglycerides; Vit.A: vitamin A; Vit.E: vitamin E; 3HB: 3-hydroxybutyrate; TP: total protein; Alb: albumin; AG: albumin/globulin ratio.

In the HF breeding herd, 8-OHdG/Cre levels were examined in eight cows at five time points from August 2022 to August 2023. Mean values (± SEM) ranged from 39.17 ± 2.12 to 76.21 ± 14.24, excluding one cow with extremely high levels. Statistical analysis revealed significant differences across sampling intervals (p = 0.01), with significant increases between August and November 2022 (p = 0.01), and higher levels in September and November 2022 than in August 2023 (p = 0.01 and p = 0.00, respectively). **Figure 5a,b** shows the mean 8-OHdG/Cre levels in the HF breeding herd and non-contaminated control herd, which showed no significant differences (p = 0.99). The P4 levels in the HF herd evaluated in August, September, and November 2022 showed statistically significant differences (p < 0.05), with a significant increase from August to September and in November (p < 0.05). **Figure 5c** presents the P4 levels (mean ± SEM) for each sampling period in the HF breeding herd.

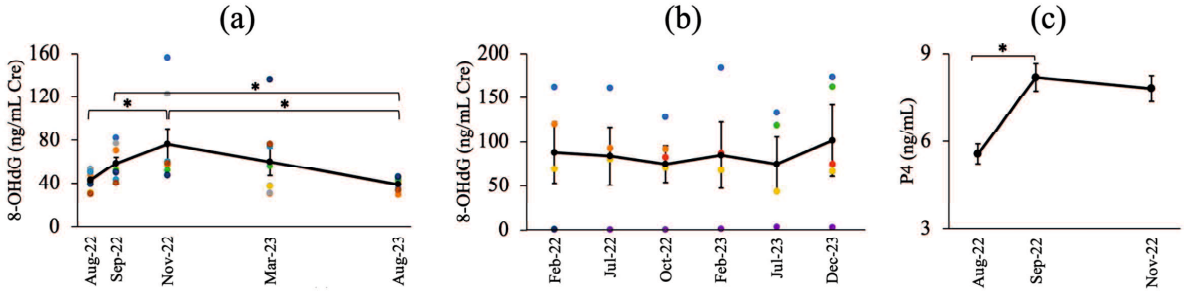

Figure 5. (a) The mean value of urinary 8-OHdG/Cre levels in the HF breeding herd (3 times dilution); (b) The mean value of urinary 8-OHdG/Cre levels in a non-contaminated breeding herd (20 times dilution); (c) Progesterone levels in the HF breeding herd at each sampling period; *: significant differences (p < 0.05). Different color dots represent the urinary 8-OHdG/Cre levels in different cows.

Clinical Information

Clinical data for the JB and HF breeding herds are shown in **Figure 6**. For herd JB (**Figure 6a**), CCRs were monitored from January 2022 to March 2023, with the lowest CCR reported in June 2022 (18.75%) and the highest in October 2022 (87.5%). In response to contamination, a mycotoxin adsorbent was added to the feed from mid-July to late November. Three miscarriages occurred in cows inseminated between November–December 2022 and February 2023 at 7–8 months of gestation. For the HF herd, clinical information was recorded from March 2022 to March 2023, and mycotoxin contamination was verified from July to November 2022. The CCRs before, during, and after contamination were 39.82%, 37.84%, and 35.77%, respectively, with the lowest CCR (14.3%) being reported in September. The highest abortion rate (22.92%) occurred before

contamination and peaked in April (28.8%). **Figure 6b** shows the CCR and abortion rates for the HF herds. **Figure 6c** shows the somatic cell count (SCC), incidence of mastitis, and treatment rates. SCC levels showed an increasing trend. The levels before, during, and after contamination were 6.17×10^4, 7.92×10^4, and 8.35×10^4, respectively. The incidence of mastitis was highest during contamination (17.5%), decreasing to 13.97% post-contamination. The treatment rate peaked at 4.72% during contamination, compared to 2.2% pre-contamination and 2.57% post-contamination.

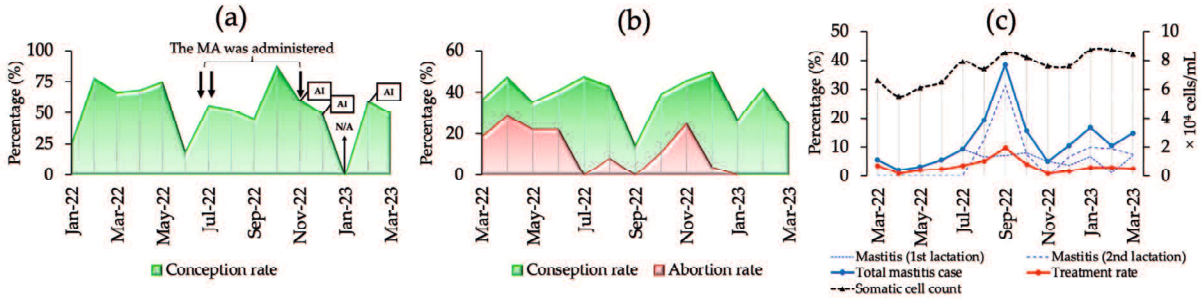

Figure 6. The clinical information of the JB and HF breeding herds. (a) The cow conception rate (CCR), feeding modification, and the date of artificial insemination (AI) in the JB breeding herd; (b) the CCR and abortion rate in the HF breeding herd; (c) the SCC, mastitis cases, and treatment rates in the HF breeding herd; N/A: the data record was not available. MA: mycotoxin adsorbent; ↓↓: the urine indicates a high level of ZEN and DON; ↓: the urine indicates a high level of DON.

DISCUSSION

Global warming has increased mycotoxin contamination in cattle feed, prompting extensive research on three key components. The first focuses on the characteristics, toxicity, and metabolites of mycotoxins in vitro and in vivo [Reisinger *et al.,* 2019; Kinkade *et al.,* 2021]. The second examines the presence of mycotoxins, often found with other fungi, in animal food and body fluids such as serum, urine, and milk [Lee *et al.,* 2018; Panasiuk *et al.,* 2019; Vandicke *et al.,* 2019; Nualkaw *et al.,* 2020]. The third area encompasses feed management, fungal infection control, and physical, chemical, enzymatic, and biological degradation methods to reduce harmful effects on animals [Liew *et al.,* 2018; Li *et al.,* 2020]. Ensilaged grass or hay may contain mycotoxins from pre-harvest contamination by *Fusarium* spp. and post-harvest contamination by fungal species commonly found in silage [Fink-Gremmels, 2008]. The co-contamination of feed with ZEN and DON, including the metabolites, is common in farms [Usleber et al., 1992; Thapa et al., 2021; Muñoz-Solano and González-Peñas, 2023]. The clinical signs of mycotoxin exposure vary depending on the trichothecene type, dose, species, route, and exposure type [Polak-Sliwinska *et al.,* 2021]. Treatments are limited to symptomatic and supportive care with prevention focused on avoiding exposure [Polak-Sliwinska *et al.,* 2021]. A comprehensive database is crucial for understanding the effect of multi-mycotoxin contamination on reproduction and productivity and for monitoring remediation responses. This study investigated the effects of ZEN and DON co-contamination on cattle health and productivity, using herds naturally affected by these contaminants as models. The JB herd represented the ZEN-dominant model, whereas the HF herd represented the DON-dominant model. Continuous monitoring revealed significant effects on health, metabolism, and reproductive capabilities, with DON-dominated contamination significantly impairing liver function that persisted even after remediation.

We successfu[6]ly identified two contaminated herds during the hot season in July, when catt[6]e are more susceptible to mycotoxin contamination. Although the herds were affected by different mycotoxins, this presented an opportunity for a novel field study. The veterinarian for JB herds reported decreased monthly pregnancy rates and improper corpus luteum development during pregnancy diagnoses, prompting urinary ZEN monitoring. Our study confirmed ZEN contamination with DON as a sub-contaminant in the feed (TMR). Simultaneous contamination of *Fusarium* spp. with ZEN and DON prompted us to investigate urinary DON measurement systems. We used a commercially available ELISA

kit to measure DON contamination, which was similar to our established urinary ZEN monitoring system [Takagi et *al.,* 2011; Widodo *et al.,* 2022]. Previous studies have reported the concurrent presence of ZEN and DON in animal feeds [Usleber *et al.,* 1992; Thapa *et al.,* 2021; Muñoz-Solano and González-Peñas, 2023]. Therefore, we developed a system to measure DON in urine samples in conjunction with our urinary ZEN monitoring method. The HF herd served as a model for identifying DON-dominant contaminants in feed.

The HF herd manager reported increased cases of clinical mastitis, reduced milk output, more abortions, lower reproductive viability, and odd feed smells, leading to the suspicion of DON contamination. The herd primarily consumed homegrown dent corn silage, and clinical symptoms in similar herds during warmer weather suggested mycotoxin exposure. Two laboratories measured feed mycotoxin levels and found significant differences in the results (**Table 2**). Mycotoxin concentrations vary according to the collection location [Fushini *et al.,* 2014; Bryden, 2012]. If only one laboratory reported DON levels below the contamination limit, DON contamination might not be suspected as the cause of mastitis or reduced fertility.

DON exposure is assessed through the analysis of urine and feces, with approximately 20% of ingested DON being recovered as 96% deepoxy-deoxynivalenol (DOM-1) and 4% DON [Côté *et al.,* 1986]. Antibody-antigen immunoassays are ideal for routine mycotoxin detection [Polak-Sliwinska *et al.,* 2021], and routine immunoassessments are necessary for effective cattle risk management [Polak-Sliwinska *et al.,* 2021; Widodo *et al.,* 2023]. Given the time and cost associated with measuring DON, we used commercially available ELISA kits to detect DON contamination in cattle herds. While immunochemical chromatography of DON is simple and affordable, its cross-reactivity and matrix effects must be explored. Advances in biomarker research have enabled the determination of DON and its metabolites in urine using single or multiple indicators [Polak-Sliwinska and Paszczyk, 2021]. DON and its acetylated derivatives 3-acetyldeoxynivalenol (3-Ac-DON) and 15-acetyldeoxynivalenol (15-Ac-DON) are produced by plants. In mice, 3-Ac-DON exposure causes oxidative damage, cell death, immune ce[6]l infiltration, and impaired liver function [Jia *et al.,* 2021]. We used ELISA kits with antibodies that cross-reacted with both DON and 3-Ac-DON, showing cross-reactivity rates exceeding 100%. Studies reported cross-reactivity levels of 520 ± 110 for 3-Ac-DON and 80 ± 10 for deoxynivalenol-3-glucoside (DON-3G) [Nguyen *et al.,* 2019]. Significant DON metabolite contamination in the tested samples indicated that the results reflected total toxin levels, not just DON levels. Thus, the kit served as a group detection tool for DON, 3-Ac-DON, and DON-3G, rather

than solely for DON [Nguyen *et al.,* 2019]. Recent reports on 3-Ac-DON toxicity, in conjunction with DON, suggest that monitoring both DON and 3-Ac-DON as "total DON" in bovine urine is beneficial. Based on preliminary recovery tests, with or without β-glucuronidase incubation, we recommend using ELISA kits to screen urinary DON concentrations. These results should be validated with LC-MS/MS, and ongoing monitoring of the DON-contaminated herd is essential to establish a comprehensive DON measurement system.

One goal was to validate our ZEN and DON detection systems using ELISA and LC-MS/MS methods. These results indicated a significant need for a biological material measurement system, particularly for urine samples. **Figure 2** shows a robust and statistically significant relationship between the ELISA and LC-MS/MS results for the two herds. Mycotoxin contamination of feed within herds is critical for herd management. Herd managers should consider the potentially harmful effects of mycotoxin contamination on the health and products of cattle. In the worst-case scenario, all contaminated feed must be disposed. Prompt identification of potential mycotoxin contaminants, assessment of cleanup efficacy, and ongoing herd monitoring are essential. Thus, we conveniently evaluated ZEN and DON contamination levels using an ELISA system to measure DON in urine, alongside a previously established ZEN concentration system. Both urinary ZEN and DON contaminations were measured simultaneously. DON levels were measured during the first 120 min of ZEN incubation. This assessment included determination of the concentration and contamination ratios of each mycotoxin, as shown schematically in **Figure 7**. This is the first practical validation of urinary ZEN and DON monitoring systems in dairy and beef herds.

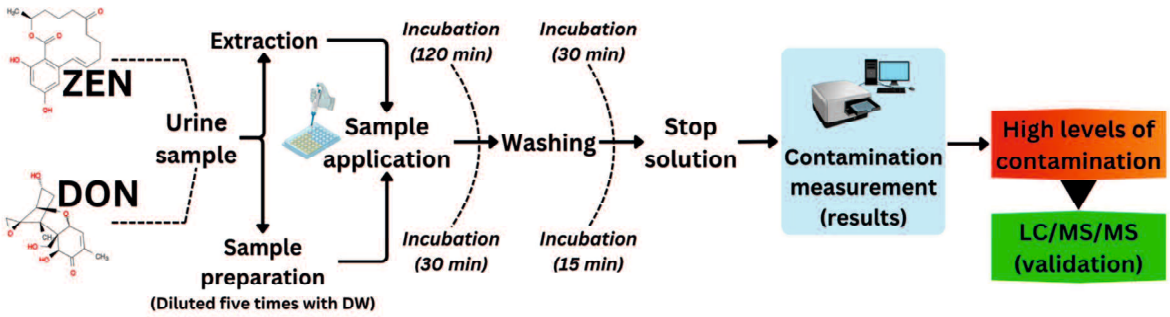

Figure 7. Flow chart of the urinary ZEN-DON monitoring system by ELISA for initial detection and LC-MS/MS for confirmation; DW: distilled water.

The greatest advantage of using urine samples to monitor the herd mycotoxin contamination status, as previously reported [Widodo *et al*., 2022, 2023], is the ability to

monitor and compare the concentrations actually ingested and absorbed from the intestinal tract. The intake volume of contaminated feed may affect the urinary mycotoxin concentration, making it a suitable method for monitoring and comparing mycotoxin exposure in cattle whose daily feed amount remains constant across each herd. To investigate the effects of chronic mycotoxin exposure on the health status and productivity of livestock herds, a urinary mycotoxin monitoring system is required to monitor mycotoxin intake from dietary feed [Widodo *et al.,* 2022]. This study revealed that simultaneous screening for ZEN-DON, which is frequently reported, is a crucial strategy to assess mycotoxin contamination in cattle herds. ELISA and LC-MS/MS concentration measurements suggested that ELISA-based urinary ZEN-DON assays may be accurate, simple, and effective for evaluating mycotoxin penetration kinetics and long-term chronic exposure at low doses.

The purpose of this study was to clarify the effects of mycotoxin exposure using serum biochemical analysis to monitor the general metabolic status after subsequent remediation. The DON-dominant herd (HF herd) exhibited significant changes in various biochemical parameters as the urinary DON concentration decreased, indicating improvements in liver function and nutritional status. However, the monitoring period showed significant changes in almost all the parameters, albeit within normal physiological ranges. GOT and GGT, indicators of liver function, significantly decreased after the counter-measures but remained elevated above normal ranges. We observed significant differences in some parameters in the ZEN-dominant (JB herd) group but did not observe any significant differences in indicators of liver function and nutritional status. Therefore, our results clearly indicated that the adverse effects on liver function were severe in DON-contaminated cattle, and in ZEN-contaminated cattle, as reported previously [Fushimi *et al.,* 2014], there appeared to be no effect on liver function (GOT or GGT). Although there are few reports on the effects of ZEN-DON and its metabolites on hematological and serum biochemical parameters in cattle under farm conditions, the effects of Fusarium toxin intake (mainly ZEN and DON) on the serum biochemical parameters of protein metabolism and liver damage in cows have been reported [Seeling *et al.,* 2005]. *Fusarium* toxin contamination of dietary feed (0.09 mg ZEN/kg DM [dry matter] and 8.21 mg DON/kg DM) was insufficient to induce toxicological changes in serum hematological variables such as GOT, GGT, TP, and Alb. Clinical manifestations vary depending on the specific trichothecene, dosage, method of exposure, and type of exposure. Compared to pure toxins, spontaneous or experimental exposure to field-contaminated materials may yield different effects [Polak-Sliwinska and

Paszczyk, 2021]. More databases are needed to determine how ZEN-DON co-contamination affects the biochemical profiles of cow herd blood.

Urinary 8-OHdG concentrations were higher in cattle predominantly contaminated with DON (herd HF). However, the concentration in the non-contaminated herd (control herd) did not change significantly, indicating that heat stress was not the cause of this increase (**Figure 1** shows monthly temperatures). We hypothesize that oxidative damage caused DNA damage during DON contamination. Moreover, our findings suggest that heat stress has no impact on the 8-OHdG assay and that there are differences in the concentration of this biomarker among various cow breeds. To the best of our knowledge, no previous studies have reported the levels of 8-OHdG in the urine of mycotoxin-contaminated herds. In addition, we found that DON-dominated contamination caused oxidative DNA damage in cattle. Further investigations and data collection are necessary to gain a comprehensive understanding of the oxidative damage induced by mycotoxins in animals. Interestingly, in the HF herd, one cow had an extremely high urinary 8-OHdG concentration during the experimental period. Our observation of changes in the clinical manifestations in this cow may be an intriguing attempt to clarify the clinical utility of 8-OHdG concentration.

Recent research on in vitro cultures has provided detailed insights into the effects of DON and its metabolites on cow follicular development and herd fertility [Pizzo *et al.,* 2016 Guerrero-Netro *et al.,* 2020]. We hypothesized that certain effects on cow reproduction could be identified by evaluating the levels of mycotoxins and reproductive performance in the two contaminated herds. To verify this, we analyzed the reproductive performance (monthly conception rate) of the two herds over a period of 4–6 months before and after the implementation of the mycotoxin contamination management strategy. **Figure 6** shows that our monitoring system detected mycotoxin contamination in the ZEN-dominant JB herd shortly after the conception rate decreased. In the DON-dominant HF herd, the monthly abortion rate remained high (20–30%) for several months, but decreased early when mycotoxin contamination was detected. From October to November 2022, we observed an increase in the abortion rate in the HF herd, potentially linked to increasing urinary ZEN concentrations, despite a decrease in urinary DON concentrations. Clinical results from these model herds revealed that elevated feed mycotoxin concentrations can significantly affect herd fertility rates, and that early contamination control measures can help herds recover faster.

In the ovary, AMH inhibits the recruitment of primordial follicles into the pool of developing follicles and decreases ovarian FSH responsiveness [Rico *et al.,* 2009, 2011].

AMH is the most reliable endocrine marker of small antral gonadotropin-responsive follicles [Rico *et al.,* 2009]. Several studies have associated ZEN and its metabolites with granulosa cell death and follicular atresia [Minervini *et al.,* 2006; Zhu *et al.,* 2012; Zhang *et al.,* 2018; Li *et al.,* 2020]. We found that natural ZEN exposure may influence AMH concentration and antral follicle count [Fushimi *et al.,* 2014]. Additionally, our previous study revealed significant correlation between urinary ZEN and blood AMH and SAA concentrations in a JB breeding herd, determined based on monthly monitoring over a 2-year period. Our findings revealed a negative correlation between urinary ZEN concentrations two months earlier and AMH concentrations in the current month [Widodo *et al.,* 2023]. Despite the lack of significant differences in AMH concentrations for each period in the ZEN-dominant JB herd, AMH kinetics were the same as in our earlier results when ZEN contamination occurred. Similarly, in the case of the DON-dominant HF herd, the AMH levels in August 2022 decreased during the two subsequent sampling periods, in September and November 2022. Subsequently, AMH levels increased again in March 2023 and peaked in August 2023. The kinetics of AMH were similar to those of ZEN. On the other hand, **Figure 3** illustrates the relationship between ZEN and SAA and DON and SAA. This shows that an increase in ZEN and DON levels leads to a subsequent increase in SAA levels, and vice versa.

However, we observed an interesting negative regression trend between ZEN and SAA concentrations in the ZEN-dominant herd (JB herd). This case showed a different trend of SAA concentrations compared to the DON-dominant herd (HF herd), with no increase in SAA concentration during feed contamination, but a decrease after contamination measures, as shown in **Figure 3b**. We previously reported an association between ZEN and SAA during the monitoring of ZEN-contaminated herds [Widodo *et al.,* 2022]. Reports indicate that ZEN not only disrupts the endocrine system but also causes immunotoxicity [Pistol *et al.,* 2013; Bulgaru *et al.,* 2021]. Other studies have shown that ZEN exposure alters the immune response of liver cells and inhibits the release of IL-1, IL-6, and TNF-α [Pistol *et al.,* 2013; Bulgaru *et al.,* 2021; Lee *et al.,* 2021]. Therefore, the negative regression trend between urinary ZEN and SAA concentrations observed in this study supports our previous report and may be attributable to innate immunosuppression in cattle resulting from acute ZEN exposure. In the future, it will be necessary to increase the number of cow herds monitored, broaden the extent of monitoring, and clarify whether improving feed while detecting naturally exposed herds facilitates an improvement in productivity.

We previously reported that ZEN concentrations exceeding 1 mg/kg in the culture medium may reduce the meiotic competence of bovine oocytes, although it does not affect

fertilization or subsequent development after in vitro fertilization [Takagi *et al.,* 2008]. Natural-feed ZEN contamination below the highest level allowed by Japanese law (<1 mg/kg) did not affect the development of superovulating embryos in JB and HF cows [Takagi *et al.,* 2013]. ZEN-contaminated feed affected cattle fertility by affecting uterine embryonic development after implantation. In the present study, the average P4 levels in the DON-dominant HF herd (30 cows) were significantly different ($p < 0.05$) during August, September, and November 2022 (**Figure 5c**), with a significant increase in P4 levels ($p < 0.05$) from August to September and November. This study did not include information on the cows' estrous cycles or pregnancies. Administering 250 mg of purified ZEN orally to heifers for three estrous cycles led to a conception rate of 62%, compared to 87% in the control heifers, and oral administration of 500 mg of ZEN for two estrous cycles resulted in normal serum P4 levels [Weaver *et al.,* 1986[a] and 1986[b]]. As mentioned previously, spontaneous or experimental exposure to field-contaminated materials may provide results different from those of purified toxins [Polak-Sliwinska and Paszczyk, 2021]. Apoptosis in granulosa cells of ZEN- and DON-contaminated herds [Guerrero-Netro *et al.,* 2015; Li *et al.,* 2020] suggests that luteinogenesis defects induce a decrease in P4 concentration during the peak of ZEN and DON contamination, which in turn leads to an increase in abortions. The results supported the presence of luteal dysplasia, as noted by the managing veterinarian during the high incidence of abortions in the ZEN-dominant (JB) herd. This is the first study to address this issue; however, more field investigations are required to understand how mycotoxins affect the luteinizing potential, P4 concentrations after follicular ovulation, and pregnancy in mycotoxin-contaminated herds.

Clinical records have demonstrated that ZEN-DON contamination affects not only fertility, but also the occurrence of mastitis. In the present study, farm managers of the DON-dominant HF herd observed an increasing clinical trend of mastitis. Exposure to grass silage containing high amounts of DON causes toxic syndrome in cattle, characterized by an increase in inflammatory reactions in the form of mastitis and laminitis [Fink-Gremmels, 2008]. Furthermore, milk yield decreases and somatic cell counts increase following exposure to moldy silage containing measurable amounts of ZEN and DON [Fink-Gremmels, 2008]. DON has been reported to inhibit lymphocyte proliferation in cattle, with the greatest decrease observed in bovine peripheral blood mononuclear cells (PBMC). It was speculated that DON might also pose a risk to the immune systems of animals typically considered to possess low susceptibility to DON, such as ruminants. This effect suggests that under certain conditions, such as inflammation or gastric diseases, low dietary DON

concentrations could potentially impair animal heart health by acting as an immunosuppressive agent [Novak *et al.,* 2018]. Recent in vitro research has shown that DON affects the proliferation and functional differentiation of bovine mammary alveolar cells, possibly by disrupting tight junctions and causing morphological alterations [Zhao *et al.,* 2022]. **Figure 6c** shows that the incidence of mastitis was 17.5% during periods of DON contamination; however, after implementing measures to address the contaminated feed, it decreased to 14.0% and further dropped to a low of 4.1%. However, the SCC increased continuously during the monitoring period, indicating that mastitis caused by DON-dominant contamination may have a prolonged effect on the SCC in dairy herds. Even at low levels, co-contamination can pose a health problem because of the possible additivity, antagonism, or synergy of their effects [Pinotti *et al.,* 2016; Muñoz-Solano and González, 2023]. Given its significant effect on dairy herd productivity, further research in this area is necessary.

The urinary ZEN and DON monitoring system is a valuable tool for identifying contaminated herds and assessing both the short- and long-term impacts of multiple contaminants on herd production and fertility under real field conditions. Implementing this system for herd management could raise awareness among managers and help prevent mycotoxin exposure in cattle herds. Although mycotoxin contamination poses a serious problem in practical herd management, it can be addressed by removing the contaminated feed (as in the ZEN-dominated JB herd) or by reducing its quantity (as in the DON-dominated HF herd). To minimize exposure, we recommend the prompt establishment of novel and accessible measures in production, such as utilization of toxin-binding agents and inactivated feed additives. In conclusion, ZEN-DON contamination adversely affects the productivity, reproductive performance, and health of breeding cattle. Implementing a urinary ZEN-DON monitoring system can be a valuable tool not only for detecting contaminants in cattle production but also for ensuring the safety of food products.

SUPPLEMENTARY MATERIAL

Table S1. Post-hoc comparison analysis of urinary ZEN-DON levels by ELISA and LC-MS/MS in the HF breeding herd

ELISA method								
Comparison	ZEN/Cre				DON/Cre			
	Mean dif.	SE	*t*-value	*p*-value	Mean dif.	SE	*t*-value	*p*-value
2022/Aug - 2022/Sep	1,204.9	2,602.5	−0.463	0.989	−5.1	43.0	−0.12	1
2022/Aug - 2022/Nov	−3,793.3	2,904.3	−1.306	0.693	235.1	29.5	7.982	0
2022/Aug - 2023/Mar	6,363.9	1,433.6	4.439	0.006	247.4	29.7	8.338	0
2022/Aug - 2023/Aug	6,755.2	1,422.3	4.75	0.004	252.6	31.1	8.123	0
2022/Sep - 2022/Nov	−2,588.4	3,413.1	−0.758	0.939	240.3	32.7	7.352	0
2022/Sep - 2023/Mar	7,568.8	2,295.5	3.297	0.056	252.5	32.9	7.682	0
2022/Sep - 2023/Aug	7,960.1	2,288.5	3.478	0.043	257.7	34.2	7.544	0
2022/Nov - 2023/Mar	10,157.2	2,632.8	3.858	0.026	12.2	10.0	1.221	0.74
2022/Nov - 2023/Aug	10,548.5	2,626.6	4.016	0.021	17.5	13.7	1.276	0.71
2023/Mar - 2023/Aug	391.3	720.6	0.543	0.981	5.2	14.1	0.37	0.99
LC-MS/MS method								
Comparison	ZEN/Cre				DON/Cre			
	Mean dif.	SE	*t*-value	*p*-value	Mean dif.	SE	*t*-value	*p*-value
2022/Aug - 2022/Sep	0.033	0.819	0.04	1	−1.416	0.576	−2.459	0.12
2022/Aug - 2022/Nov	1.93	0.819	2.356	0.15	1.749	0.576	3.039	0.03
2022/Aug - 2023/Mar	3.98	0.819	4.859	0	1.978	0.576	3.436	0.01
2022/Aug - 2023/Aug	0.941	0.819	1.149	0.78	1.833	0.576	3.185	0.02
2022/Sep - 2022/Nov	−1.897	0.84	−2.257	0.18	3.165	0.591	5.359	0
2022/Sep - 2023/Mar	−3.947	0.84	−4.697	0	3.394	0.591	5.746	0
2022/Sep - 2023/Aug	−0.908	0.84	−1.081	0.81	3.249	0.591	5.501	0
2022/Nov - 2023/Mar	−2.050	0.84	−2.439	0.12	0.229	0.591	0.387	0.99
2022/Nov - 2023/Aug	0.989	0.84	1.177	0.76	0.084	0.591	0.142	1
2023/Mar - 2023/Aug	3.039	0.84	3.616	0.01	−0.145	0.591	−0.245	0.99

Cre: creatinine; Mean dif.: mean difference; SE: standard error

Table S2. Results of blood biochemical analyses (mean ± SEM) of the JB and HF breeding herd

JB breeding herd						
Parameters	Sampling date					*p*-value
	2022/Jul	2022/Aug	2022/Oct	2023/Nov	2023/Dec	
GOT#	67.25 ± 2.65	54.37 ± 2.62	60.96 ± 3.27	58.3 ± 2.39	57.46 ± 4.49	0.061
GGT	15.88 ± 1.62	14.29 ± 0.98	14.92 ± 1.04	15.98 ± 2.18	18.74 ± 3.33	0.454
FFA*	159.57 ± 15.05	213.83 ± 26.44	275.87 ± 42.98	213.78 ± 40.51	97.78 ± 22.84	0.024
T-Cho*	137.4 ± 11.11	107.48 ± 3.4	97.65 ± 6.36	113.14 ± 5.56	130.8 ± 8.5	0.002
BUN*	11.23 ± 0.41	10.31 ± 0.58	8.58 ± 0.42	8.6 ± 0.45	9.78 ± 0.41	0.004
Glu*	67.36 ± 0.93	58.65 ± 1.46	60.67 ± 2.53	60.5 ± 2.99	62.82 ± 0.72	0.043
Ca#	9.38 ± 0.15	9.32 ± 0.13	9.52 ± 0.15	9.06 ± 0.18	8.78 ± 0.28	0.067
IP*	7.7 ± 0.28	6.48 ± 0.24	5.48 ± 0.32	4.84 ± 0.22	5.1 ± 0.13	0.000
Mg	1.98 ± 0.06	2.03 ± 0.07	2 ± 0.04	2.06 ± 0.1	2.08 ± 0.12	0.878
TG*	15.2 ± 1.63	28.37 ± 2.46	21.71 ± 2.32	12.44 ± 2.56	16.56 ± 2.99	0.000
Vit. A*	163.11 ± 6.95	106 ± 3.12	119 ± 4.52	131.4 ± 4.17	122.6 ± 3.31	0.000
Vit. E*	198.22 ± 20.17	180.67 ± 12.15	199.85 ± 22.6	304.6 ± 29.93	326.8 ± 41.88	0.000
3HB*	448.36 ± 27.77	443.26 ± 28.36	330.68 ± 18.63	290.36 ± 34.16	577.4 ± 48.26	0.000
TP*	6.4 ± 0.16	8.04 ± 0.18	7.29 ± 0.23	8 ± 0.3	7.62 ± 0.26	0.000
Alb	3.45 ± 0.06	3.43 ± 0.07	3.42 ± 0.08	3.574 ± 0.06	3.48 ± 0.04	0.72
AG*	1.19 ± 0.05	0.77 ± 0.05	0.94 ± 0.07	0.834 ± 0.08	0.86 ± 0.06	0.000
HF breeding herd						
Parameters	Sampling date					*p*-value
	2022/Aug	2022/Sep	2022/Nov	2023/Mar	2023/Aug	
GOT*	123.30 ± 5.01	106.27 ± 3.61	94.43 ± 4.64	101.06 ± 4.63	111.37 ± 8.88	0.000
GGT*	46.51 ± 3.27	39.21 ± 2.18	32.82 ± 1.73	34.52 ± 3.08	41.67 ± 5.31	0.000
FFA*	190.16 ± 5.65	167.75 ± 14.30	182.89 ± 13.03	230.65 ± 50.89	185.97 ± 20.22	0.030
T-Cho*	208.84 ± 7.66	213.20 ± 6.10	218.52 ± 7.99	249.25 ± 14.69	267.28 ± 15.77	0.000
BUN*	11.49 ± 0.43	15.37 ± 0.56	13.73 ± 0.48	13.29 ± 0.58	9.67 ± 0.55	0.000
Glu*	17.78 ± 1.64	43.57 ± 1.53	67.21 ± 0.82	56.27 ± 1.29	63.56 ± 0.95	0.000
Ca*	9.72 ± 0.08	9.37 ± 0.12	9.17 ± 0.26	9.03 ± 0.19	9.02 ± 0.07	0.000
IP*	7.07 ± 0.12	6.20 ± 0.17	5.98 ± 0.18	6.54 ± 0.13	5.46 ± 0.13	0.000
Mg*	2.50 ± 0.04	2.36 ± 0.04	2.46 ± 0.04	2.55 ± 0.09	2.53 ± 0.04	0.020
TG*	6.92 ± 0.34	6.70 ± 0.38	7.97 ± 0.65	6.34 ± 0.52	12.58 ± 2.14	0.000
Vit. A*	155.60 ± 3.55	197.13 ± 32.65	160.83 ± 5.35	167.78 ± 7.43	130.54 ± 4.06	0.000
Vit. E	577.53 ± 28.49	615.96 ± 26.04	670.83 ± 24.01	633.74 ± 38.39	640.50 ± 32.22	0.240
3HB*	746.58 ± 40.85	773.03 ± 67.71	387.92 ± 22.71	544.06 ± 48.03	691.30 ± 32.67	0.000
TP	7.32 ± 0.07	7.26 ± 0.08	7.30 ± 0.08	7.28 ± 0.10	7.13 ± 0.07	0.390
Alb*	3.84 ± 0.04	3.79 ± 0.04	3.62 ± 0.04	3.87 ± 0.07	3.88 ± 0.04	0.000
AG*	1.13 ± 0.04	1.12 ± 0.03	1.00 ± 0.03	1.17 ± 0.05	1.22 ± 0.04	0.000

*= statistically significant result ($p < 0.05$); #= significant tendencies (p-value = 0.05–0.10)

OVERAL DISCUSSION AND CONCLUSION

Various studies have shown the harmful effects of mycotoxin exposure on humans and animals. One health recognizes that the health of humans, animals, and ecosystems is interconnected. Our research focuses on naturally occurring mycotoxin contamination in feed, which affects cattle health and reproduction. We aim to ensure a mycotoxin-controlled food supply chain. 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 and reproductive biomarkers [Takagi *et al*., 2011; Hasunuma *et al*., 2012; Fushimi *et al*., 2014]. This thesis discusses the practical application of our established ZEN urinary monitoring system and the development of a novel DON monitoring system utilizing commercially available kits for cattle herds. This technology could serve as a mycotoxin prevention method at the farm level, particularly in beef cattle and dairy cattle production.

In Chapters 1 and 2, we performed monthly monitoring on a JB herd naturally contaminated with ZEN and STC. We performed monthly monitoring over a span of 25 months (long-term monitoring), presented the first-year data in Chapter 1, and analyzed the long-term monitoring data in Chapter 2. The urinary mycotoxin monitoring system offers a significant advantage, as the digestive tract directly ingests and absorbs the mycotoxin concentration. Conversely, the sampling point in a feed sample significantly influences the detectable level of contamination. Chapter 1 describes the measurement results of ELISA and LC-MS/MS, the two measurement methods used for ZEN concentration. Simultaneous screening of cattle herds within the same geographical area with similar breeding environments will be an important future strategy to understand the prevalence and status of mycotoxin contamination in cattle populations. The ELISA approach provides accurate, useful, and efficient results for measuring ZEN in urine. This method effectively demonstrates how mycotoxin infiltration changes over time and at low concentrations. Furthermore, our results indicated that a low ZEN value one month prior may tend to result in a high AMH value in the current month, and a low ZEN value in the current month suggested a tendency for higher AMH values in the next month. The adverse effects of ZEN on AMH secretion appeared early, but recovery of AMH secretion after ZEN exposure may need a considerable duration. Additionally, a high ZEN value may tend to result in a low SAA value in the sampling month. The negative regression trend between urinary ZEN and

SAA concentrations obtained in the present study is possibly due to the innate immune suppression of cows from prolonged low-level exposure to ZEN.

Chapter 2 elucidates ZEN contamination in relation to AMH and SAA. Following a year of observation (Chapter 1), we identified the trends in the relationship between urinary ZEN and AMH concentrations, as well as between urinary ZEN and SAA concentrations. Chapter 2 of the current investigation demonstrated significant co-relationships among the urine concentrations of ZEN, AMH, and SAA in the breeding herd. During the monitoring period, we observed a shorter calving interval, a key indicator for assessing cattle reproductive performance. Ultimately, our monitoring provided novel insights about AMH levels in aging JB cows. Reports indicate that multiparous cows exhibit higher AMH concentrations throughout the postpartum period compared to primiparous cows. In our findings, the AMH levels of fixed cows were evaluated, revealing that while AMH levels fluctuated with varying urinary ZEN concentrations, there was a statistically significant decline in AMH levels as the cows aged. This may indicate a decline in the number of antral follicles in aged cows. Additional research utilizing data from more cows is necessary for clarifying this phenomenon.

As described in Chapter 3, our study focuses on the effects of mycotoxin co-contamination (ZEN and DON) on major biomarkers, including AMH, SAA (blood biochemistry), urinary 8-OHdG, and P4 levels. Clinical data indicate that ZEN-DON contamination impacts both fertility and the incidence of mastitis. We examined the impact of co-contamination by ZEN and DON on cattle health and productivity, using herds naturally exposed to these contaminants as models. The JB herd represented the ZEN-dominant model, whereas the HF herd represented the DON-dominant model. In this study, we developed a DON urinary monitoring system using commercially available ELISA kits, which included antibodies that cross-reacted with both DON and 3-Ac-DON, demonstrating cross-reactivity rates exceeding 100%. The kit served as a group detection tool for DON, 3-Ac-DON, and DON-3G. We also validated our ZEN and DON detection systems using ELISA and LC-MS/MS methods, suggesting that ELISA-based urinary ZEN-DON assays may be accurate, simple, and cost-effective for evaluating mycotoxin penetration kinetics.

In conclusion, the urinary ZEN and DON monitoring system is a valuable tool for identifying contaminated herds and assessing both the short- and long-term impacts of multiple contaminants on herd production and fertility under real field conditions. Using a urinary ZEN and DON monitoring system for herd management could potentially raise awareness among herd managers and prevent mycotoxin exposure in cattle herds. Zen-Don

contamination adversely affects the productivity, reproductive performance, and health of breeding cattle. The implementation of a urinary ZEN-DON monitoring system can serve as a valuable tool in ensuring the safety of food supply chains.

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