

The effects of zearalenone on embryo quality in
Japanese black and Holstein cows and the
monthly variation of zearalenone concentration
in TMR and urine samples during seasonal
changes in cattle.

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Abstract

Fusarium species make multiple mycotoxins of which the most important are deoxinivalenol (DON) and zearalenone (ZEA). ZEA and its metabolites α -zearalenol (α -ZOL) and β -zearalenol (β -ZOL) have reportedly an effect on the reproductive system because they act as an estradiol agonist. Chronic DON exposure in cattle can reduce phagocytic and neutrophilic activity.

The objective of the first experiment was to determine if there was any correlation between the embryo quality after superovulation treatment and ZEA concentration in the urine of Japanese black and Holstein cattle. The urine ZEA concentration was determined using an ELISA kit, superovulation treatment and embryo flushing was done without any additions. Quality of the embryos was defined as the percentage of transferable embryos, percentage degenerated embryos and percentage unfertilized embryos. The ZEA concentration in the urine was measured because this has proven to be a biomarker for ZEA exposure (Takagi et al. 2011). A commercially available ELISA kit was used because this is a reliable method to monitor the urine ZEA concentration (Takagi et al. 2011). De ZEA concentration is expressed as the ration to urinary creatinine (Crea).

No significant correlations were observed between the urinary ZEA concentration of Japanese Blacks and the total number of embryos $r=-0,015$ $P=0,956$, transferable embryos $r=0,191$ $P=0,479$, degenerated embryos $r=-0,108$ $P=0,689$ or unfertilized embryos $r=-0,372$ $P=0,156$. In the Holstein group also no significant correlations were discovered. Holstein ZEA concentration and the total number of embryos $r=0,053$ $P=0,815$, transferable embryos $r=0,011$ $P=0,960$, degenerated embryos $r=0,141$ $P=0,532$ or unfertilized embryos $r=-0,015$ $P=0,948$.

In the second experiment the ZEA concentration in the TMR and urine was checked monthly. The goal was to determine a correlation and/or significant difference in the ZEA in TMR and urine throughout the year. During this research the same ELISA kit was used to determine the ZEA but also the DON concentration. The third and final objective was to determine if there was a correlation between the SSC and DON concentration. SSC data were collected from the farm records to find a correlation with the DON. No DON was detected in the urine and TMR samples by the ELISA and LC/MC/MC. The individual urinary ZEA concentration by itself had no correlation with the individual SSC in the Holstein herd $r=-0.027$ $P=0.86$.

In this experiment no correlation was found between the urinary and TMR samples that were collected the same month $r=0,167$ $P=0,604$. However, there was a significant difference in the Urinary ZEA concentration between December and April, December and July $P<0,05$. Nevertheless this result is questionable because of many variables decreasing the standardization. The two experiments were conducted under field conditions to examine the effect of these 2 mycotoxines.

Introduction

Mycotoxins are secondary metabolites produced by fungi and can potentially be present in the feed of animals. Due to a mycotoxins contamination a conceivably adverse economic effect can occur depending on the nature of the mycotoxin (Bryden 2012). The effects of the intake of mycotoxins can be acute or chronic, by which an acute intake usually results in a high mortality rate. In most cases the concentration of mycotoxins is not so high as to cause acute effect (Bryden 2012).

Mycotoxins are often present in feed of animals but usually cause chronic effects like growth retardation, reduced reproductive performance and immune suppression (Bryden 2012). The effects of the mycotoxins vary widely and depend on the type of toxin. In this respect, the concentration levels of the toxin depend on a large number of variables: "physical factors (moisture, relative humidity, temperature and mechanical damage), chemical factors (carbon dioxide, oxygen, composition of substrate, pesticide and fungicides), and biological factors (plant variety, stress, insects, spore load) (Bryden 2012)". Moreover, many different factors contribute to the degree of sensitivity to mycotoxins (see Figure 1). The impacts of physiological and environmental factors are different for each species and even between individuals of the same species.

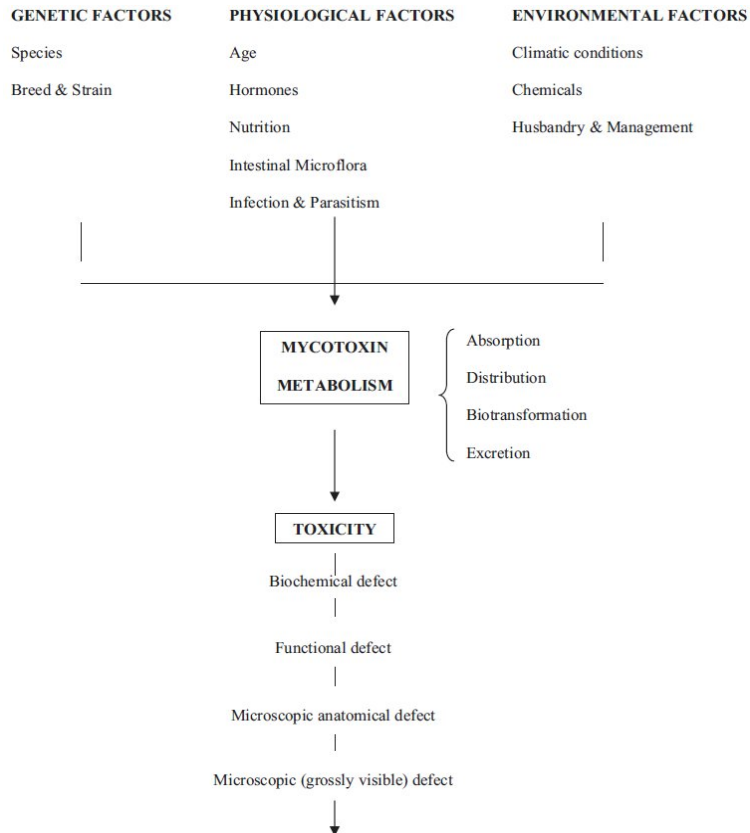


Figure 1: Different factors contributing to less or more sensitivity to mycotoxins (Bryden 2012).

For example, Holstein cows in Texas are housed in a very different environment compared to Holstein kept in the Netherlands. Many of these factors contribute to the concentration levels of the mycotoxins the animal will be exposed and to what extent the animal will respond after ingestion. For example the micro flora in the rumen of ruminants will degrade most of the mycotoxines keeping the concentrations below toxic levels (Zinedine et al. 2007). Finally, species specific sensitivity to mycotoxins has been shown, for example pig and rabbits are very sensitive to the toxic effects of mycotoxins (Liu et al. 1985)

This paper will mainly focus on Zearalenone (ZEA). ZEA or 6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic acid lactone is a secondary metabolite and a non-steroidal estrogenic mycotoxin produced by a *Fusarium graminearum*, *F. culmorum*, *F. crookwellense*, *F. equiseti* and *F. semitectum* (Rossi et al. 2009). These fungi species can be present in maize, rice wheat, barley and other crops (FinkGremmels and Malekinejad 2007). *F. graminearum*, *F. culmorum* are also associated with the production of deoxynivalenol (DON) (see table 1). Often a combination of DON and ZEA can be found in feed because they are often associated with the same *Fusarium* species (see figure 1).

Chronic DON exposure in cattle can reduce phagocytic and neutrophilic activity and may result in lower IgA antibody levels (Korosteleva 2007, Smith & Boermans 2007).

ZEA is metabolized in the body, mainly in the intestines and the liver (FinkGremmels and Malekinejad 2007). The phase 1 metabolic step of this mycotoxin is a hydroxylation reaction that reduces the keto group at C-6'. This reaction produces the important metabolites of ZEA, α -zearalenol (α -ZOL) and β -zearalenol (β -ZOL) (FinkGremmels and Malekinejad 2007, Rossi et al. 2009). This reaction is catalyzed by 3 α - and 3 β -hydroxysteroid-dehydrogenase (HSD). If α -ZOL and β -ZOL are reduced α - and β -zearalanol are formed; these metabolites however are usually not measured. ZEA, α -ZOL and β -ZOL exert their effect by binding to the estrogen receptor (ERs) acting as a competitive agonist/antagonist and presumably activate gene transcription at both type of ERs (FinkGremmels

and Malekinejad 2007). A difference in α -ZOL/ β -ZOL conformation ratio was found between animal species (Hassan Malekinejad, MaasBakker & FinkGremmels 2005): α -ZOL was predominant in pigs and humans, β -ZOL in poultry and ruminants (FinkGremmels and Malekinejad 2007). This difference is because ruminants and poultry mainly express 3β -HSD and pigs and human 3α -HSD, at target tissue sites (FinkGremmels, Malekinejad 2007). Ruminants and poultry are less affected by ZEA, since at concentration levels that have been shown adversely affect pigs almost no effect was observed in ruminants and poultry.

ZEA and its metabolites exert an estrogenic effect through binding to the estrogen receptors. ER α receptors have been found in the germinal epithelium, interstitial cells and theca cells (Beker-van Woudenberg et al. 2004). ER β receptors have been detected in granulosa- and cumulus cells (Beker-van Woudenberg et al. 2004). During meiotic maturation of the oocyte proper spindle formation is a prerequisite. The structural changes during the process of spindle formation are associated with the reorganization of microtubules and microfilaments which are the most predominant components of the cytoskeleton in cattle oocytes that eventually distribute the sister chromosomes evenly across the cells. In correct microtubule formation and function in cells will gain or lose chromosomes resulting in aneuploidy (Beker-van Woudenberg et al. 2004). Aneuploid cells will give abnormal development after fertilization. In an experiment where in vitro oocytes were matured in a culture environment that contained too high concentrations of estradiol, 35% of the oocytes showed impaired spindle malformations at 22 h after maturation (Beker-van Woudenberg et al. 2004). Thus overstimulation with estrogen or estrogen agonists like ZEA, α -ZOL or β -ZOL may inhibit oocyte nuclear maturation caused by chromosome spindle malformation (Bekervan Woudenberg et al. 2004, J. Fink-Gremmels 2008).

Ultimately, this finding could have negatively consequences for fertility of animals that are chronically exposed to ZEA in feed. In cattle artificial insemination or embryo production by superovulation are the most used reproductive technologies to achieve pregnant cows (Velazquez 2011). Both technology are widely used in breeding programs to reduce the transmission of venereal diseases and costs (Mapletoft and Hasler 2005). To the best of our knowledge no report has described the possible relationship between the yield and quality of bovine embryos derived after superovulation treatment and the intake of feed naturally contaminated with mycotoxins.

The study consisted of three main objectives. The first objective (experiment 1) was to determine the effect of ZEA concentration measured in the urine on the total number of embryos and the quality of the embryos collected after a superovulation treatment under field conditions.

The second objective (experiment 2) was to determine a possible relationship between ZEA concentrations in the total mix ration (TMR) fed to dairy cows and the monthly variation of ZEA measured in the urine, especially variations related to seasonal effects under standard farm and field conditions. The third objective (experiment 3), was to determine a relationship between the DON concentration in the TMR and the somatic cell count (SSC).

Table 1: An overview of the corresponding mycotoxins produced by different Fungal species (Bryden 2012).

Fungal species	Mycotoxin
<i>Aspergillus flavus</i> ; <i>A. parasiticus</i>	Aflatoxins
<i>A. flavus</i>	Cyclopiazonic acid
<i>A. ochraceus</i> ; <i>A. carbonarius</i> ; <i>Penicillium verrucosum</i>	Ochratoxin A
<i>P. citrinum</i> ; <i>P. expansum</i>	Citrinin
<i>Fusarium sporotrichioides</i> ; <i>F. poae</i>	T-2 toxin
<i>F. sporotrichioides</i> ; <i>F. poae</i>	Diacetoxyscirpenol
<i>F. culmorum</i> ; <i>F. graminearum</i>	Deoxynivalenol
<i>F. culmorum</i> ; <i>F. graminearum</i>	Zearalenone
<i>F. verticillioides</i> ; <i>F. proliferatum</i>	Fumonisin
<i>Alternaria alternata</i>	Tenuazonic acid
<i>Claviceps purpurea</i>	Ergot alkaloids

Material and Methods

Animals

For experiment 1, 38 cows were used as test subjects. Of these thirty-eight cows there were sixteen Japanese Black and twenty-two Holstein cows. The cows were distributed over twenty-five farms in Hokkaido prefecture Japan. Every cow was fed according to the Japanese Feeding Standard for Japanese Black or Holstein. There was unlimited access to fresh water for all animals. Before the start of the experiment the body condition score (BCS) and age were both determined by a skilled veterinarian. BCS was ranged from a scale from 1 to 5 where 1 is thin and 5 obese, all the cows were between 3 and 3,5. Japanese black cows had an average age of $4,3 \pm 2,8$ the Holstein cows $5,2 \pm 3,2$.

In experiment 2, a herd with twenty Holstein cows was used from a farm in Yamaguchi prefecture. As in experiment 1, the cows were fed according to the Japanese feeding standard for Holstein dairy cattle and had unlimited access to fresh water. The average body weight was 650kg and 40kg milk on average was produced per cow per day. 12kg TMR feed was provided daily for all the cows, who were randomly assigned to tie stalls with individual feeders.

Experimental design and Samples collection

Experiment 1: The superovulation stimulation was executed according to the standard protocol. Injections of follicle stimulating hormone (FSH; Antrin, Kyoritsu Seiyaku corp., Tokyo Japan) were administered on days 8 to 12 (estrus = Day 0), twice daily with decreasing dose. Injections were given at half-day intervals. 40 AU FSH was given in total to the Holstein cows and 20 AU FSH to the Japanese Black cattle (see table 2).

Japanese black cattle started on the 8th day of the cycle with 2 doses of 5AU FSH, the Holstein cattle got two doses of 10 AU FSH on the 8th day of the cycle. The 9th day, 3AU FSH at half-day interval was administered to the Japanese Black and 5 AU FSH for the Holstein cows.

On the 10th day the Japanese black got 2 AU and Holstein 4 AU FSH. Because of the difference in species and the total dose of FSH between Japanese Black and Holstein the cows were divided into two groups.

Table 2: Superstimulation treatment schedule

Day (estrus = Day 0)	Japanese Black	Holstein
8	2 x 5AU FSH at 12 hour interval	2 x 10AU FSH at 12 hour interval
9	2 x 3AU FSH at 12 hour interval	2 x 5AU FSH at 12 hour interval
10	2 x 1AU FSH at 12 hour interval and prostaglandin	2 x 3AU FSH at 12 hour interval and prostaglandin
11	2 x 1AU FSH at 12 hour interval	2 x 2AU FSH at 12 hour interval
13 /14	2x AI	2x AI
20	Embryo Flushing	Embryo Flushing

A Prostaglandin $F_{2\alpha}$ analogue ($PGF_{2\alpha}$; Pronalgon F) was administered together with the 5th dose of FSH on the tenth day of the estrus cycle inducing luteolysis (see table 2). On day 13 the artificial insemination (AI) was performed, 24 hours later a second dose of sperm was administered for insemination (see table 2). Frozen sperm was used for the AI and 2 dose straws per cow were used. The quality of the used sperm is unknown at this time. On day 20 the embryos were flushed from the uteri horns. The horns were flushed with Reflux flushing media heated to 38,5 degrees Celsius. To the reflux washing media fetal calves serum was added to prevent the embryos sticking.

After retrieving the fluid flushed from the uterus it was directly inspected to determine the viability of the embryos. The embryos were evaluated for: total number of embryos, transferable embryos, degenerated embryos, unfertilized embryos and the stage of embryonic development. Criteria for the evaluation included: Regularity of shape of the embryo, compactness of the blastomeres,

variation in cell size, color and texture of the cytoplasm, overall diameter of the embryo, presence of extruded cells, and regularity of the zona pellucida.

Embryos in with more than 25% of total cell mass were considered viable and whose development was not severely impaired were considered transferable. Checking the embryos was done under a microscope with 10-14 times magnification. Each embryo was checked twice and washed with reflux media. This was all preformed on a 38,5 degrees Celsius hotplate. After all embryos were checked they were immediately used or cryopreserved. After embryo recovery, from each cow a urine sample was collected by spontaneous urination after massage of the pudendum. The urine samples were stored in a cooler ranging from 5 – 8 degrees Celsius to prevent contact with sunlight. After arrival in the laboratory the samples were stored in a freezer at minus thirty degrees Celsius.

Experiment 2: Urine samples were collected 3 hours after the morning feeding round from all cows at the start of each month. The farmer collected this himself by vaginal massage. Also 1 kg of the TMR was collected simultaneously. All the urine samples were frozen directly to prevent any chemical change and were transported in a cooling box (-10 to -15°C) without contact to sunlight. In the laboratory the samples were kept at minus thirty degrees Celsius. SSC's numbers were systematically acquired from the farm records. The farmer routinely checked the SSC of every cow using an automatic milk component analyzer (FOSS Electric). There are 46 measurements of ZEA concentration and their overlapping SSC.

Elisa analysis

Experiment 1: A commercial available ELISA kit was used for the experiments to measure the urinary ZEA concentration (RIDA SCREEN Zearalenon, R-Biopharm AG, Darmstadt, Germany). First the ZEA and its metabolites have to be extracted from the urine. A 0.5 ml urine sample got diluted with 3 ml 50mM acetate buffer with a pH of 4,8. Then 8 µl glucuronidase was added and the samples were stored overnight in a 37 degrees Celsius water bath.

Next RIDA® C18 columns were used with a flow rate of 1 drop/sec. First the columns were rinsed with 3ml 100% ethanol, then to equilibrate the column 2 ml 20mM Tris buffer (pH 8,5)/ methanol (80%/20%) was added. Next 3,5 ml of the sample was added. This was followed by 2 ml of Tris buffer and 3 ml methanol 40%

The columns were dried by centrifuge at 500g for 10 minutes. Then 2 ml of ethanol was added to elute the samples with a flow rate of 15 drops/min. Eluate was evaporated at 60 degrees Celsius. The dried residue was redissolved with 50 µl methanol and 450 µl sample dilution buffer. 50µ of this mixture with the extracted ZEA and its metabolites was used for the ELISA. Finally RIDA SOFT win software was used to compute the absorbance at 450nm on the microplate spectrophotometer. Based on three trials the ELISA's mean recovery rate was determined as 84% ± 14%. The ZEA concentrations were not adjusted (Takagi et al. 2011).

The Crea concentrations were also measured using an ELISA kit (Sikarait-S CRE, Kanto Chemical). The Crea ELISA kit was preformed according to the guidelines, after the ELISA the Crea was determined with a clinical autoanalyser (7700 Clinical Analyzer). The urine ZEA concentration is expressed as ratio to the urinary Crea concentration (ZEA/CREA (pg/mg)). This was done to correct for different concentrations of urine.

Experiment 2: The same ELISA kit was used to measure the ZEA and DON concentration in the urine and TMR samples (RIDA SCREEN Zearalenon and Deoxynivalenol, R-Biopharm AG, Darmstadt, Germany).

The TMR samples were homogenized and of each sample 5g aliquot sample was taken. To this aliquot sample 25 ml 70% methanol was added and the samples were vortexed vigorously for 3 min and stored for 24 hours at room temperature. After 24 hour, filter paper (Filter paper A5) was used to filter the samples. The filtrate was used for the ELISA (same ELISA kit as in experiment 1) to ascertain the ZEA and DON concentration. Also here the urinary ZEA concentration is expressed as the ratio to the urinary Crea concentration. The ZEA concentration in the feed is displayed as parts per billion. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was used for the TMR samples taken in February, May and July to confirm the results from the ELISA. The LC/MS/MS was

used at Shokukan Inc. in Gunma Prefecture Japan, the mean recovery rate for ZEA was 80% and 90% for DON.

Statistical methods

For both experiments the statistical computer program Statistical Package for the Social Sciences (SPSS version 16) was used, P values <0.05 were considered to be statistically significant.

Experiment 1: The Pearson correlation coefficient was used to determine correlation between de urinary ZEA concentration and the total number of embryos, transferable embryos, degenerated embryos and unfertilized embryos. Also the percentages of transferable embryos (number of transferable embryos divided by total number of embryos) were calculated and compared between the different breeds. This was used for the correlation between the ZEA concentration and transferable percentage of embryos and was plotted in a scatter plot.

Experiment 2: The mean and standard error of the mean (SEM) for ZEA, DON and SSC were calculated. The Pearson's correlation coefficient was used to calculate the correlation and significance between the mean monthly ZEA concentration and mean monthly SCC of the cows (n = 46). The Pearson correlation was also used to calculate the correlation between the urinary ZEA concentration and TMR ZEA concentration.

Results

Experiment 1: Table 7 and table 8 display the individual zearalenone concentration in comparison to the total number of embryos, transferable embryos, degenerate embryos and unfertilized embryos for the Japanese black respectively Holstein cows. In the present study we divided the cattle in to 2 groups. Group 1 consisted of 16 Japanese Black cows and group 2 consisted of 22 Holstein Cows. The mean ZEA concentration was 97,4 pg/mg Crea and standard deviation (SD) of $\pm 43,4$ for Japanese Black and $155,5 \pm 43,4$ pg/mg Crea for Holstein (see table 7 and 8). The maximum concentration that was found is 404,5 pg/mg Crea in one of the Holstein cows (table 8). No significant correlations were observed between the urinary ZEA concentration of Japanese Blacks and the total number of embryos $r=-0,015$ $P=0,956$, number of transferable embryos $r=0,191$ $P=0,479$, degenerated embryos $r=-0,108$ $P=0,689$ or unfertilized embryos $r=-0,372$ $P=0,156$ (see Table 3). The correlation between the urinary ZEA concentration and the percentage of transferable embryos is also seen in figure 3

Table 3: Japanese Black ZEA correlation with total, transferable, degenerated and unfertilized embryos

		ZEA	Total embryos	Transferable embryos	Degenerated embryos	Unfertilised embryos
Urinary ZEA Concentration Japanese Black	<u>Pearson Correlation</u>	1	-,015	,191	-,108	-,372
	Sig. (2-tailed)		,956	,479	,689	,156
	N	16	16	16	16	16

Table 4: Holstein ZEA correlation with total, transferable, degenerated and unfertilized embryos

		ZEA	Total embryos	Transferable embryos	Degenerated embryos	Unfertilised embryos
Urinary ZEA Concentration Holstein	<u>Pearson Correlation</u>	1	,053	,011	,141	-,015
	Sig. (2-tailed)		,815	,960	,532	,948
	N	22	22	22	22	22



Figure 2: Scatter plot of the percentage (%) of transferable Holstein embryos and the ZEA concentration pg/mg

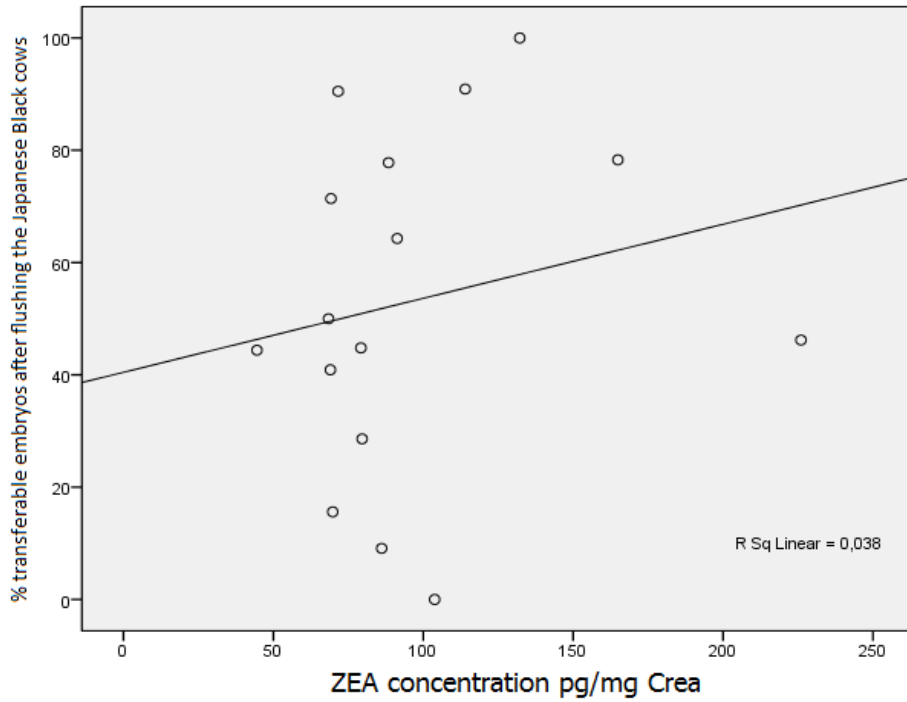


Figure 3: Scatter plot of the percentage (%) of transferable Japanese Black embryos and the ZEA concentration pg/mg

In the Holstein group no significant correlations were discovered either. Holstein ZEA concentration and the total number of embryos $r=0,053$ $P=0,815$, transferable embryos $r=0,011$ $P=0,960$, degenerated embryos $r=0,141$ $P=0,532$ or unfertilized embryos $r=-0,015$ $P=0,948$ (see Table 4). Figure 2 shows the percentage of transferable embryos and the corresponding ZEA concentration. Also here it is illustrated that there is no significant correlation.

Experiment 2: No DON was detected in the urine and TMR samples by the ELISA and LC/MC/MC. The urinary and TMR ZEA concentrations from the samples taken each month are shown in figure 4. ZEA and DON have not exceeded the permissible levels in the TMR, permissible level for ZEA is 1ppm. The individual urinary ZEA concentration by itself had no correlation with the individual SSC in the Holstein herd $r=-0.027$ $P=0.86$ (see table 5). No correlation was observed between the urinary and TMR samples that were collected the same month $r=0,167$ $P=0,604$ (see table 6). There was however a significant difference in the Urinary ZEA concentration between December and April, December and July $P<0,05$ (see figure 4).

Table 5: Correlation between urinary ZEA concentration and SSC

		Urinary ZEA concentration	Somatic cell count values
Urinary ZEA concentration	Pearson Correlation	1	-,027
	Sig. (2-tailed)		,860
	N	46	46

Table 6: Correlation between urinary ZEA concentration and TMR ZEA concentration

		Mean ZEA Concentration	TMR ZEA concentration ppb
Mean ZEA concentration per month	Pearson Correlation	1	,167
	Sig. (2-tailed)		,604
	N	12	12

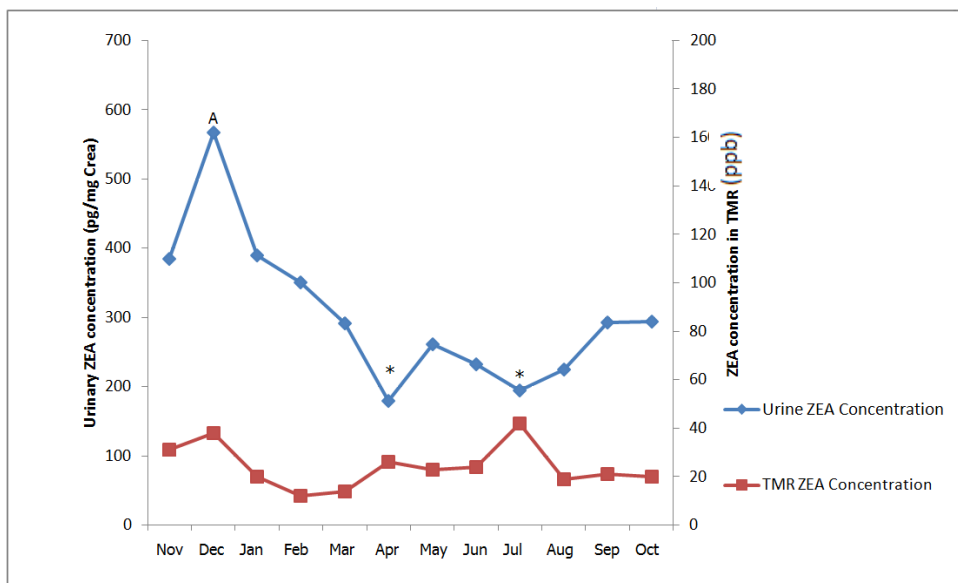


Figure 4: ZEA concentration in monthly Urine and TMR. Significant difference between A and * ($P<0,05$)

	Breed	ZEA Con	Total	Trans	Degen	Unfer	Trans %	Degen %	Unfer %
1	JB	44,5	9	4	2	3	44,4	22,2	33,3
2	JB	91,3	14	9	4	1	64,3	28,6	7,1
3	JB	86,1	11	1	5	5	9,1	45,5	45,5
4	JB	79,6	7	2	3	2	28,6	42,9	28,6
5	JB	69,0	22	9	10	3	40,9	45,5	13,6
6	JB	68,4	10	5	0	5	50,0	0,0	50,0
7	JB	88,4	18	14	3	1	77,8	16,7	5,6
8	JB	71,6	21	19	2	0	90,5	9,5	0,0
9	JB	69,8	32	5	24	3	15,6	75,0	9,4
10	JB	79,2	29	13	16	0	44,8	55,2	0,0
11	JB	69,2	7	5	2	0	71,4	28,6	0,0
12	JB	103,8	1	0	0	1	0,0	0,0	100,0
13	JB	114,0	22	20	2	0	90,9	9,1	0,0
14	JB	132,2	10	10	0	0	100,0	0,0	0,0
15	JB	164,9	23	18	3	2	78,3	13,0	8,7
16	JB	226,0	13	6	7	0	46,2	53,8	0,0
	Mean±SD	97,4 ±43,4	15,6±8,35	8,8±6,21	5,2±6,29	1,74±1,69	53,3±30,4	27,8±22,9	18,9±27,4

Table 7: The individual Japanese black (JP) cattle zearalenone concentration (pg/mg Crea) in relationship to the total number of embryos, transferrable embryos (trans), degenerate embryos (degen) and unfertilized embryos (Unfer)

	Breed	ZEA Con	Total	Trans	Degen	Unfer	Trans %	Degen %	Unfer %
1	HF	82,1	9	9	0	0	100,0	0,0	0,0
2	HF	32,7	1	0	0	1	0,0	0,0	100,0
3	HF	76,1	22	18	4	0	81,8	18,2	0,0
4	HF	43,8	17	11	4	2	64,7	23,5	11,8
5	HF	87,5	22	10	5	7	45,5	22,7	31,8
6	HF	88,1	17	16	0	1	94,1	0,0	5,9
7	HF	106,1	13	13	0	0	100,0	0,0	0,0
8	HF	110,0	8	7	1	0	87,5	12,5	0,0
9	HF	105,7	8	7	1	0	87,5	12,5	0,0
10	HF	105,6	5	5	0	0	100,0	0,0	0,0
11	HF	105,6	12	3	9	0	25,0	75,0	0,0
12	HF	134,2	9	8	1	0	88,9	11,1	0,0
13	HF	117,8	17	13	4	0	76,5	23,5	0,0
14	HF	146,9	27	15	12	0	55,6	44,4	0,0
15	HF	201,0	13	9	2	2	69,2	15,4	15,4
16	HF	262,6	2	2	0	0	100,0	0,0	0,0
17	HF	256,2	11	7	4	0	63,6	36,4	0,0
18	HF	221,7	4	1	3	0	25,0	75,0	0,0
19	HF	242,3	6	3	0	3	50,0	0,0	50,0
20	HF	276,2	49	37	8	4	75,5	16,3	8,2
21	HF	213,3	10	2	8	0	20,0	80,0	0,0
22	HF	404,5	9	6	3	0	66,7	33,3	0,0
	Mean±SD	155,5±92,03	13,2±10,4	9,2±7,98	3,1±3,45	0,9±1,77	67,1±22,9	22,7±25,4	10,1±23,6

Table 8: The individual Holstein (HF) cattle zearalenone concentration (pg/mg Crea) in relationship to the total number of embryos, transferrable embryos (trans), degenerate embryos (degen) and unfertilized embryos (Unfer)

Discussion

Experiment 1

Effects of ZEA on the reproductive system have been reported numerous times in many different species (FinkGremmels and Malekinejad 2007, Takagi et al. 2011, Zinedine et al. 2007, Rossi et al. 2009). However only a fraction of the research papers on the fertility of cattle are under field conditions and field exposure to ZEA. Using an ELISA kit is an effective method to measure the ZEA concentration in urine (Takagi et al. 2011). Previous research of Takagi et al. 2011 also showed that naturally contaminated feed can be effectively monitored by measuring the urinary ZEA concentration with the ELISA kit (Takagi et al. 2011). Using this information we could conclude that in this experiment obtained urinary ZEA concentration, indicates that the cows received TMR with ZEA concentrations within the acceptable limit. The maximum of ZEA in TMR set by the government in Japan is 1000 µg/kg (1 ppm).

The obtained results may partially support our thesis because the feed was naturally contaminated with *Fusarium* and did not exceed the permissible amount of the Japanese government. Thus ZEA concentrations that do not exceed permissible concentrations in the feed did not have any adverse effects on the total number of embryos or embryo quality.

However, there is also a difference in susceptibility of cattle to ZEA in comparison with non-ruminant species. This could be explained through individual differences due to physiological factors (age, breed etc.) and the ability of the flora, which leads to degrading of ZEA in the rumen (J. Fink-Gremmels 2008). Even though our experimental group did show any effects to these ZEA concentrations it is likely that pigs, who are more sensitive to ZEA, could experience negative effects regarding the embryo quality.

As discussed earlier, the effect of ZEA is lower in ruminants due to degrading by the flora in the rumen (J. Fink-Gremmels 2008). However there is also research suggesting that ZEA has indeed effects on the fertility of ruminants. This effect however is only observed after administrating high concentrations of ZEA to ruminants. In an experiment where a controlled amount of ZEA was given to ewes the ovulation rate, cycle length and estrus duration were decreased (J. Fink-Gremmels 2008, Smith, Menna & McGowan 1990). The diet containing the ZEA had apparently no effect on the rams because neither the volume nor quality of the semen was different (Smith, Menna & McGowan 1990).

After feeding heifers 250 mg crystalline ZEA per day, conception rates decreased from 87% to 62% (Weaver et al. 1986). Examining the reproductive organs and hormone concentrations in the blood did not result in significant observed changes (Weaver et al. 1986). Other researchers indicated that ZEA causes hyperoestrogenism in cattle (FinkGremmels and Malekinejad 2007, J. Fink-Gremmels 2008). In another experiment 1.25 mg ZEA/kg feed was given in the feed to heifers, but no histological or pathological changes were observed in the reproductive organs (Moeser 2001).

Hormone concentrations did not change either and the cycle did not differ (Moeser 2001). In an *in vitro* experiment cumulus-oocyte complexes from pigs were exposed to ZEA, DON and 17 β -estradiol (all 3.12 µmol/L) (Hassan Malekinejad 2007). The presence of high concentration 17 β -estradiol reduced the number of oocytes that reached meiose 2 stage significantly reduced. Spindle malformation in the oocyte was observed with very high oestradiol concentration (Hassan Malekinejad 2007). Presence of ZEA (3.12 µmol/L) during maturation reduced the percentages of oocytes that cleaved and formed a blastocyst to about 12%, compared with 25% of control oocytes.

The experiments described above administer an extremely high dose of ZEA to the animals or *in vitro* to the oocytes. It is known that high estradiol concentrations can impair oocyt maturation and cause spindle malformations during meiosis (Bekervan Woudenberg et al. 2004). However it was not known if ZEA or α -ZOL and β -ZOL as estrogen agonist could exert the same effects *in vivo* in ruminants. In our experiment the highest ZEA concentration was 404.5 pg/mg creatinine. The concentration of ZEA, α -ZOL and β -ZOL in our own experiment were not significant enough to induce any effect in the embryos.

Our experiment did not detect any correlation between the ZEA and the embryos quality and total number of embryos. However the quality of the sperm that was used is not known. This factor also contributes to the quality of the transferable embryos and of course the amount of fertilized embryos. Furthermore the animals were divided in two groups, in Holstein and Japanese Black, because of the different amount of FSH given for the superovulation treatment. There is also a difference in diet, weight, genetics etc. but the difference in FSH could have effects on the total number of embryos and the quality of each embryo.

Moreover the experiment was conducted on 25 different farms and 2 different breed of cows, which makes it very difficult to account for all the variables between every farm. Determining the effect of ZEA and its metabolites in this experiment is suboptimal. Ideally the study would have been conducted on 1 farm with known concentrations of ZEA and have a control group, but it was one of the objectives to research in field conditions. Conducting the experiment on 25 different farms might also suggest that even though there were many different farms and variables, the ZEA concentration under the legally permissible amount will have no significant effect on the embryo quality.

Thus the present study may indicate that the ZEA concentrations within the permissible amounts will not affect embryo quality and total number of embryos in standard farm environment. For further research it is perhaps necessary to count the follicles by echo before and after the embryo flushing. Using echo equipment will provide more information on the development or changes of the follicles and ovaries.

Experiment 2

Factors contributing to the growth of fungi are physical factors such as moisture, relative humidity, temperature. Especially moisture and temperature contribute to fungi growth and mycotoxin production (Bryden 2012). Due to the importance of these physical factors on fungus growth the objective of the second experiment was to determine if fusarium mycotoxines differ in the feed with the changing climate and seasons throughout the year. In colder seasons the urinary ZEA concentration was high especially in December. Concentration of ZEA in the TMR sample of December does not correlate with the urinary concentration. The results show that there was a significant difference in urinary ZEA concentration between December and April and December and July (see figure 4). Differences in season and climate could account for the differences observed. However the TMR ZEA concentration does not correspond with the high urinary ZEA concentration, thus it is questionable how significant the concentration in December is. Unfortunately it is unknown under what conditions the plants were grown: for example if there was insect damage or drought, how much the pre-harvest was contaminated, if the feed was stored differently throughout the year. Furthermore the method of sample collection for the TMR is unknown. Ideally this was done at random and a mix sample was taken. These many unknown factors contribute to the low standardization of the experiment, making it impossible to determine the precise effects of the seasonal changes on the ZEA concentration in the TMR and urine.

The results however can partially support the thesis because the feed was natural contaminated with Fusarium and did not exceed the permissible amount of the Japanese government. With a natural contamination of Fusarium in the feed, seasonal changes do not contribute to significant changes for the ZEA concentration.

With our current method it is furthermore possible for farmers to check the ZEA concentration effortlessly and cost effectively in the feed of the cattle. The ELISA kit is not expensive and thus may provide a simplified method for every farmer to send in samples to determine the ZEA concentration for monthly monitoring. In the future the ELISA technique for determining ZEA concentrations could very well ensure improved food safety of cattle feed.

The third objective was to determine a correlation between DON and the SSC. No DON was detected in any of the samples with the ELISA or LC/MS/MS. So we can't conclude that DON has any effect on the SSC. In scientific literature it is reported that DON can reduce phagocytic and neutrophilic activity. More research is necessary to determine the exact effect of DON in cattle. For further research ideally a controlled amount of DON should be given to an experimental group of cattle. Also a control group is necessary to get more significant results.

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