

## The level of zearalenone and $\alpha$ -zearalenol in the blood of gilts with clinical symptoms of toxicosis, fed diets with a low zearalenone content\*

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### ABSTRACT

Twenty-four immature hybrid gilts (Large Polish x Polish Landrace) were randomly divided into three groups in order to determine zearalenone (ZEA) and  $\alpha$ -zearalenol concentrations in blood after feeding them with feeds containing low doses of crystalline zearalenone (groups II and III, 200 and 400  $\mu\text{g kg}^{-1}$  body weight, respectively). The mycotoxin was administered in gelatine capsules once a day during the morning feeding (at 6.30) for seven consecutive days. On the first day of the experiment, 5.5 h after feeding, the concentration of ZEA was the highest in group II ( $9.43 \pm 1.32$  ng ml<sup>-1</sup>), and the concentration of  $\alpha$ -ZEA was the highest in group III in the second hour of the experiment ( $24.13 \pm 5.98$  ng ml<sup>-1</sup>). Mean concentrations of ZEA were:  $1.54 \pm 0.16$  up to  $9.92 \pm 0.80$  ng ml<sup>-1</sup> (II) and  $2.34 \pm 0.33$  up to  $10.90 \pm 0.93$  ng ml<sup>-1</sup> (III), respectively, whereas mean concentrations of  $\alpha$ -ZEA were:  $2.86 \pm 0.23$  up to  $8.11 \pm 0.60$  ng ml<sup>-1</sup> (II) and  $4.48 \pm 0.22$  up to  $17.79 \pm 2.19$  ng ml<sup>-1</sup> (III), respectively. It was found that administering a LOAEL (Lowest Observable Adverse Effect Level) dose of ZEA *per os* caused weakly manifested symptoms of hyperoestrogenism, whereas swelling and reddening of the vulva were much stronger at a dose double the LOAEL dose. There was no distinct positive correlation between the level of zearalenone and  $\alpha$ -zearalenol in blood and the occurrence of the external symptoms of hyperoestrogenism.

KEY WORDS: mycotoxins, zearalenone, hyperoestrogenism, pig

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## INTRODUCTION

Animals in the Żuławy and Lubelszczyzna regions of in Poland are consistently at high risk of fusariosis of crops caused by *Fusarium* sp. (Juszkiewicz and Piskorska-Pliszczynska, 1992). In the Warmia and Mazury region, from May 2000 to May 2001 zearalenone was found in maize in 12 out of 66 samples in amounts ranging from 12.50 to 303.00  $\mu\text{g kg}^{-1}$ ; in balanced feeds, in 8 of 23 examined samples, ranging in amounts from 8.88 to 50.70  $\mu\text{g kg}^{-1}$ , and in barley, wheat, rye and oats in amounts averaging 128.00  $\mu\text{g kg}^{-1}$  (Obremski, unpublished data). Although these mycotoxin concentrations are not very high, after a few months of improper storage, growing fungi moulds will probably increase mycotoxin production.

Zearalenone (ZEA) is a phytoestrogen mycotoxin (Farnsworth et al., 1975; Price and Fenwick, 1985; Obremski et al., 1999; Krazeisen et al., 2001) that belongs to a group of macrocyclic lactones; it is a secondary metabolite of *F. culmorum*, *F. equiseti*, *F. moniliforme* and *F. tricinctum* or *F. graminearum* (Mirocha and Christensen, 1974).

After entering the body of an animal through the digestive tract, the mycotoxin ZEA causes swelling and reddening of the vulva, histopathological changes in the ovaries and uterus (Blaney et al., 1984; Sundlof and Strickland, 1986; Vanyi et al., 1994; Curtui et al., 2001; Gajęcki, 2002), infertility, false pregnancies, histopathological changes and lactation disorders in mammary glands (Mirocha and Christensen, 1974; Etienne and Jemmali, 1982), and splayleg in piglets (Sydenham et al., 1988). The influence of zearalenone is not limited only to processes related to reproduction. It has been found to affect certain biochemical transformations connected with general metabolism, namely reactions in the respiratory chain and oxidative phosphorylation (Lundh and Lundgren, 1991). Pigs, especially gilts, are most sensitive to ZEA toxicity before the first oestrus cycle. Mirocha and Christensen (1974) report that 1 mg of ZEA administered *per os* may cause symptoms of false oestrus in pigs within 48 h after administration.

It is not unusual for pigs to consume natural zearalenone with feeds at a dose of about 200  $\mu\text{g ZEA kg}^{-1}$  of body weight daily. This value is regarded as the lowest dose causing the first observable clinical symptoms of oestrogenism in pigs, the so-called Lowest Observable Adverse Effect Level (LOAEL), (IARC, 1993). There is no data concerning blood concentrations of zearalenone and its metabolite in correlation with clinical symptoms accompanying zearalenone intoxication at the LOAEL level. The knowledge of the above values could be useful in the differential diagnosis of hyperoestrogenism in pigs.

The aim of this paper is to examine the blood levels of zearalenone and  $\alpha$ -zearalenol in sexually immature gilts displaying clinical symptoms of toxicity caused by crystalline zearalenone administered at the LOAEL.

## MATERIAL AND METHODS

*Animals, feed, zearalenone*

The experiment was carried out in compliance with all of the legal regulations binding in Poland regulating the conditions and manner in which such experiments on animals may be conducted. Twenty-four hybrid gilts (Large Polish x Polish White) were used in the study. The animals were clinically healthy, 120-125 days old, of an average body weight (BW) of  $49.2 \pm 3.6$  kg. The gilts were fed about 3 kg of feed twice a day at 6.30 a.m. and 3 p.m.; the feeds were free from zearalenone and other mycotoxins such as ochratoxin A, aflatoxins, and deoxynivalenol, as confirmed by analysis of feed samples in the Laboratory of the Department of Veterinary Prophylaxis and Feed Hygiene, Faculty of Veterinary Medicine of the Warmia and Mazury University in Olsztyn.

The animals were kept under identical conditions in separate boxes with constant water supply. The gilts were divided into three groups (n = 8) given gelatine capsules containing: I - control group, non-toxic feed (placebo); II - experimental group, receiving ZEA at a dose of  $200 \mu\text{g kg}^{-1}$  BW  $\text{day}^{-1}$ , and III, experimental group, receiving ZEA at a dose of  $400 \mu\text{g kg}^{-1}$  of BW  $\text{day}^{-1}$ .

ZEA (Zearalenone Z-0167 Sigma Chemical CO. Steinheim, Germany) 12 h before administration was dissolved in ethyl alcohol (Ethyl alcohol 96%, SWW 2442-90; Polish Chemical Reagents, SA, Gliwice, Poland) and applied in the required amount to feed, placed in a gelatine capsule and left for 12 h at room temperature allowing the solvent to evaporate. The capsules were administered once a day during the morning feeding (6.30 a.m.) for seven consecutive days.

*Procedures on animals*

In order to improve the blood sampling and to make it stress-free, a surgical operation was carried out to insert a PVC catheter, ID 1.2 and OD 1.6 mm, into the *vena cava cranialis*, according to the method described by Kotwica et al. (1978). Premedication was carried out by an intramuscular injection of atropine sulphate (Atropiona; Polfa, Warsaw) at a dose of  $0.04 \text{ mg kg}^{-1}$  of BW and propionylpromasine (Combelen; Biovet, Puławy) at a dose of  $0.2 \text{ mg kg}^{-1}$  BW. General anaesthesia was induced by intravenous (ear marginal vein) administration of sodium pentobarbitalate (Vetbutal; Biovet, Puławy) at a dose of  $10 \text{ mg kg}^{-1}$  BW. After the surgery, amoxycyclin (Betamox® L.A.; SCANVET) was administered intramuscularly at a dose of  $1 \text{ ml } 10 \text{ kg}^{-1}$  BW as protection against infection.

*Sampling*

Blood samples were taken through the previously inserted catheters; each time the loss of blood was supplemented with a similar amount of Solfin® (Polfa, Kut-

no, Poland) solution. In order to prevent clotting, the catheter was filled with 3 ml of Solfin with heparin (Heparyn Biochemie GmbH, Kunal, Austria) at a concentration of 25 000 m.u. 500 ml<sup>-1</sup> of Solfin.

In order to determine the content of ZEA and  $\alpha$ -ZEA, blood samples were taken at 8.30 a.m., 12.00 and 2.00 p.m. for seven consecutive days. Subsequently, the blood was transferred to pre-cooled centrifuge test-tubes, containing heparin and centrifuged for 20 min at 2 000 g at 4°C. The obtained serum was transferred into 3 ml plastic test-tubes and stored at -20°C.

#### *Determination of zearalenone and $\alpha$ -zearalenone in blood*

The content of ZEA and  $\alpha$ -ZEA in blood serum was measured with the use of the following separation techniques: immunoaffinity columns (Zearala-Test™ Zearalenone Testing System, G1012, VICAM, Watertown, USA) and high-performance liquid chromatography (HPLC) with fluorescence detection.

#### *Preparing serum for analysis*

Serum samples were defrosted in a water bath (LW-1 type) at 37°C and pH 5.5. To the samples were added 5 000 IU of  $\beta$ -glucuronidase (H-2 type from *Helix pomatia* with an enzymatic activity of 100 000 IU ml<sup>-1</sup> and pH 5.0, G7017 Sigma-Aldrich Sp. SA oo.) to cleave the mycotoxins from glucuronic acid and incubated for 16 h at 37°C (Olsen et al., 1985). After the incubation, 6 ml of methanol of gradient purity (LiChrosolv™, No. 1.06 007, Merck-Hitachi, Germany) were added to the sample in order to extract the toxins. The sample was vigorously stirred and allowed to stand for 20 min at room temperature and then centrifuged (MPS 370 centrifuge) for 15 min at 2 000 g. The supernatant was collected and passed through a Zearala-Test immunoaffinity column, manufactured by VICAM. In order to remove impurities, the column was washed with de-ionised water (Millipore Water Purification System, Millipore S.A., Molsheim, France) and the toxins bound with the substrate were eluted with 99.8% methanol. The obtained eluate was placed in a water bath at 50°C where all of the solvent was evaporated in a stream of nitrogen; the residue was dissolved in 0.5 ml of methanol.

#### *High-performance liquid chromatography (HPLC)*

The analysis of mycotoxins was performed with the use of a fluorescence detector (Hewlett Packard 1100, FLD G1321A), a ODS Hypersil 5  $\mu$ m 4 x 250 mm (No. 799 260D-584, Agilent, USA) chromatographic column with an RP-18 pre-column. The injection volume was 100  $\mu$ l. The mobile phase composition was established as follows: acetonitrile : methanol : water (46:8 : 46%), using acetonitri-

le (LiChrosolv™, No. 984 730 109, Merck-Hitachi, Germany), methanol (LiChrosolv™, No. 1.06 007, Merck-Hitachi, Germany) and de-ionised water (Millipore Water Purification System, Millipore S.A., Molsheim, France). The rate of the mobile phase flow was 1.8 ml min<sup>-1</sup>. The excitation wavelength, emission wavelength, temperature of the column oven and the duration of chromatographic analysis were established, as:  $\lambda_{\text{Ex}} = 218$  nm,  $\lambda_{\text{Em}} = 438$  nm, 30°C and 4 min, respectively.

The data were registered and integrated using a POL-LAB Computer Integrator and the CHROMAX for Windows ver. 2000 (Pol-Lab. Artur Dzieniszewski) computer program for processing chromatographic data. The mycotoxin concentrations were calculated by the external standard method and the values were expressed in ppb (ng ml<sup>-1</sup>).

Adding zearalenone and  $\alpha$ -zearalenol to mycotoxin-free sera showed a good rate of recovery ranging from  $82 \pm 6.7$  to  $89 \pm 1.8\%$ .

#### *Statistical analysis*

The obtained results are presented as mean  $x \pm$  standard error of mean (SEM). Bi-factor variance analysis (ANOVA) was applied for statistical verification of the results. In case of rejecting all the null hypothesis, the differences were verified with the Duncan test. The above analyses were performed with Statsoft STATISTICA® computer software.

## RESULTS AND DISCUSSION

The first oestrus in gilts occurs between the 4<sup>th</sup> and 9<sup>th</sup> month of life. Gradual reddening and swelling of the vulva and a specific body position called the tolerance reflex are the external clinical symptoms. Oestrus is the result the activity of oestrogens secreted by developing ovarian follicles (Dziuk, 1977). It is also known that progressing oedema and reddening of the vulva are not signs of oestrus but of hyperoestrogenism understood not as hyperfunction of oestrogens but as a result of triggering oestrogen receptors by other substances or oestrogen-like substances (i.e. phytoestrogens) (Krazeisen et al., 2001). Because of the phenolic ring in its structure, zearalenone can binds to oestrogen receptors (Katzenellenbogen, 1996) and can cause false oestrus symptoms. Sows and especially gilts before the first oestrus are very sensitive to zearalenone. ZEA interaction with oestrogen receptors gives not only external hyperoestrogenic symptoms (Gajęcki, 2002), but also triggers a reaction cascade increasing RNA and cell protein synthesis and reproductive tissue cell proliferation in the organs of the reproductive system (Ueno and Yagasaki, 1975). Hyperoestrogenism caused by zearalenone (Olsen et al., 1985) is very harmful to gilts for many reasons, including delayed first oestrus or lower mating efficiency (Etienne and Dourmad, 1994).

In the experiment, sexually immature gilts were given feed with crystalline zearalenone at a concentration of 200 (LOAEL) or 400  $\mu\text{g kg}^{-1}$  of BW (2 x LOAEL). The concentration of zearalenone and  $\alpha$ -zearalenol in gilt blood from control (I) and experimental (II, III) groups on the first day is given in Table 1. In the control group there were no mycotoxins in the animals' blood. In the experimental groups, 2 h after administering crystalline mycotoxin with feed, there was a higher level of ZEA in experimental group II, and the concentration of  $\alpha$ -zearalenol was three times higher in experimental group III. The second assay done 3.5 h later showed a highly statistically significant doubling of the ZEA level in group II and tripling in group III. Analysis of mycotoxin levels in the third and last measurement showed a nearly four-fold decrease in group II and only three-fold decline in group III (highly significant statistically,  $\alpha = 0.01$ ) of the ZEA concentration in comparison with the first measurement. The level of  $\alpha$ -ZEA was also higher, but in group III it was a highly significant decrease ( $\alpha = 0.01$ ) in comparison with the first measurement.

The levels of ZEA and  $\alpha$ -ZEA as an average value calculated from three daily measurements taken on consecutive days, are given in Table 2. Differences in mycotoxin levels observed during each day of the experiment in groups II and III were highly significant ( $\alpha=0.01$ ).

The average concentrations of ZEA, calculated from the three daily measurements on consecutive days of the experiment ranged in group II from  $0.10 \pm 0.08$  (day 4) to  $9.92 \pm 0.80$   $\text{ng ml}^{-1}$  (day 5). The changes in the concentration on particular days differed very significantly ( $\alpha = 0.01$ ). Average concentrations of ZEA, calculated from the three daily measurements on consecutive days of the experiment ranged in group III from  $2.34 \pm 0.33$  (day 4) to  $14.45 \pm 0.79$   $\text{ng ml}^{-1}$  (day 7). The changes in the concentration on particular days differed very significantly ( $\alpha = 0.01$ ).

The average concentrations of  $\alpha$ -ZEA on the following days of the experiment in group II ranged from  $1.08 \pm 0.07$  (day 8) to  $8.11 \pm 0.60$   $\text{ng ml}^{-1}$  (day 1), and in group III from  $2.29 \pm 0.18$   $\text{ng ml}^{-1}$  (day 8) to  $17.79 \pm 2.19$   $\text{ng ml}^{-1}$  (day 1). The changes in the concentrations on particular days were statistically significant ( $\alpha = 0.01$ ) (Table 2).

In four-month-old experimental gilts, which were given small amounts of mycotoxin, clinical symptoms of hyperoestrogenism could be seen on the 3<sup>rd</sup> and 4<sup>th</sup> day of the experiment (groups II and III, respectively). The oedema and reddening observed in them were differently intensified.  $\alpha$ -Zearalenone shows greater affinity to oestrogenic receptors, being a three times stronger oestrogen than zearalenone (Kuiper-Goodman et al., 1987). On this basis, it can be assumed that its increasing concentration is related to intensified symptoms of hyperoestrogenism. This relation was not observed in this study. Some researchers observed that after administering zearalenone with feed, the concentration of  $\alpha$ -zearalenol in serum was

TABLE 1

Hours of examinations	ZEA ng ml <sup>-1</sup>			$\alpha$ -ZEA ng ml <sup>-1</sup>		
	I	II	III	I	II	III
8.30	0.00 ± 0.00	3.52 <sup>B</sup> ± 0.19	2.39 <sup>B</sup> ± 0.24	0.00 ± 0.00	7.92 ± 0.65	24.13 <sup>A</sup> ± 5.98
12.00	0.00 ± 0.00	8.07 <sup>A</sup> ± 1.25	7.22 <sup>A</sup> ± 0.96	0.00 ± 0.00	9.43 ± 1.32	16.39 <sup>B</sup> ± 1.56
14.00	0.00 ± 0.00	0.93 <sup>B</sup> ± 0.07	2.50 <sup>B</sup> ± 0.27	0.00 ± 0.00	6.97 ± 0.97	12.84 <sup>B</sup> ± 0.67

\* <sup>A,B</sup> significance at  $\alpha = 0.01$ ; I, II and III groups - n=8; mean ± SEM

TABLE 2

Days of experiments	ZEA ng ml <sup>-1</sup>			$\alpha$ -ZEA ng ml <sup>-1</sup>		
	I	II	III	I	II	III
1	0.00 ± 0.00	4.17 <sup>C</sup> ± 0.71	4.04 <sup>CD</sup> ± 0.55	0.00 ± 0.00	8.11 <sup>A</sup> ± 0.60	17.79 <sup>A</sup> ± 2.19
2	0.00 ± 0.00	1.76 <sup>D</sup> ± 0.15	2.52 <sup>D</sup> ± 0.29	0.00 ± 0.00	7.16 <sup>A</sup> ± 0.39	13.58 <sup>B</sup> ± 1.07
3	0.00 ± 0.00	1.54 <sup>D</sup> ± 0.16	2.57 <sup>D</sup> ± 0.34	0.00 ± 0.00	6.13 <sup>AB</sup> ± 0.55	11.52 <sup>BC</sup> ± 0.39
4	0.00 ± 0.00	0.10 <sup>D</sup> ± 0.08	2.34 <sup>D</sup> ± 0.33	0.00 ± 0.00	4.15 <sup>BC</sup> ± 0.47	11.07 <sup>BC</sup> ± 0.84
5	0.00 ± 0.00	9.92 <sup>A</sup> ± 0.80	12.10 <sup>AB</sup> ± 0.79	0.00 ± 0.00	6.30 <sup>AB</sup> ± 0.63	4.53 <sup>DE</sup> ± 0.35
6	0.00 ± 0.00	7.62 <sup>B</sup> ± 0.36	10.90 <sup>B</sup> ± 0.93	0.00 ± 0.00	5.90 <sup>AB</sup> ± 0.57	8.20 <sup>EF</sup> ± 0.33
7	0.00 ± 0.00	9.05 <sup>AB</sup> ± 0.66	14.45 <sup>A</sup> ± 0.79	0.00 ± 0.00	2.86 <sup>CD</sup> ± 0.23	4.48 <sup>DE</sup> ± 0.22

\* <sup>A,B,C,D</sup> - significance at  $\alpha = 0.01$ ; I, II and III groups - n=8; mean ± SEM

higher than the concentration of zearalenol, whereas in other studies, the concentration of the maternal compound was higher than the concentration of  $\alpha$ -zearalenol (Bauer et al., 1987; Kuiper-Goodman et al., 1987). Many different factors may influence the results of these studies.

The absence of a correlation, which undoubtedly makes diagnosis difficult, may be the result of feed composition—the percentage of fibre in the diet (Olsen et al., 1991) or the enterohepatic circulation, functioning in pigs, which enables the re-circulation of zearalenone from the alimentary tract (Biehl et al., 1993).

## CONCLUSIONS

In summary, administering zearalenone *per os* at a dose of 200  $\mu\text{g kg}^{-1}$  (LOAEL) caused weakly manifested symptoms of hyperoestrogenism in sexually immature gilts as early as on the fourth day of toxin administration, whereas at a dose of 400  $\mu\text{g kg}^{-1}$  (two times LOAEL), the clinical symptoms of hyperoestrogenism were much stronger and presented a day earlier, on the third day of the study. No significant correlation between zearalenone and  $\alpha$ -zearalenol concentrations in the blood of the animals or intensification of signs of hyperoestrogenism was observed.

Probably there is no distinct positive correlation between the level of zearalenone and  $\alpha$ -zearalenol in blood and the occurrence of the external symptoms of hyperoestrogenism, because of the enterohepatic circulation in pigs, which removes a large amount of mycotoxins from the blood stream (the toxins were present in the intestinal contents and bile).

It is important to stress (in case of false oestrus in gilts the lack of tolerance reflex or low efficiency in mating) the necessity of assaying zearalenone or  $\alpha$ -zearalenol in blood in order to eliminate feed as a source of zearalenone that has not been detected by mycotoxicological examination of feed.

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## STRESZCZENIE

**Poziom zearalenonu i  $\alpha$ -zearalenolu w krwi loszek z klinicznym obrazem zatrucia po żywieniu paszami z niską zawartością zearalenonu**

Dwadzieścia cztery niedojrzałe loszki-mieszance (wielka biała x polska biała zwisłoucha) podzielono losowo na 3 grupy, celem określenia poziomu zearalenonu (ZEA) i  $\alpha$ -zearalenolu w krwi przy skarmianiu pasz zawierających małe dawki krystalicznego zearalenonu (grupa II i III, odpowiednio 200 i 400  $\mu\text{g kg}^{-1}$  m.c.). Mikotoksynę podawano w żelatynowych kapsułkach 1 raz dziennie podczas porannego odpasu (o godz. 6,30), przez 7 kolejnych dni. W pierwszym dniu doświadczenia, w 5,5 godz. po karmieniu, najwyższy poziom ZEA stwierdzono w grupie II ( $9,43 \pm 1,32$  ng  $\text{ml}^{-1}$ ), a  $\alpha$ -ZEA w grupie III w 2 godzinie badań ( $24,13 \pm 5,98$  ng  $\text{ml}^{-1}$ ). Średnia koncentracja ZEA wynosiła odpowiednio:  $1,54 \pm 0,16$  do  $9,92 \pm 0,80$  ng  $\text{ml}^{-1}$  (II) i  $2,34 \pm 0,33$  do  $10,90 \pm 0,93$  ng  $\text{ml}^{-1}$  (III), natomiast średnia koncentracja  $\alpha$ -ZEA odpowiednio:  $2,86 \pm 0,23$  do  $8,11 \pm 0,60$  ng  $\text{ml}^{-1}$  (II) i  $4,48 \pm 0,22$  do  $17,79 \pm 2,19$  ng  $\text{ml}^{-1}$  (III). Stwierdzono, że ZEA podawany *per os* w dawce LOAEL powoduje wystąpienie słabo wyrażonych objawów hiperestrogenizmu, natomiast przy dawce dwukrotnie większej od LOAEL obrzęk i zaczerwienienie sromu są o wiele bardziej nasilone. Nie wykazano wyraźnej dodatniej korelacji pomiędzy poziomem zearalenonu i  $\alpha$ -zearalenolu w krwi a występowaniem zewnętrznych objawów hiperestrogenizmu.