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Original article

The effect of selected environmental Fusarium mycotoxins on the ovaries in the female wild boar (*Sus scrofa*)

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Abstract

The contamination of agricultural crops with Fusarium mycotoxins poses one of the greatest problems in food production. Wild boars live in specific habitats and are physiologically sensitive to Fusarium mycotoxins, therefore, they are an interesting model for studies investigating the effects of the discussed toxin, in particular under low-dose exposure. The objective of this study was to determine potential effects of Fusarium mycotoxins ingested with naturally contaminated food on reproductive function based on the proliferation and apoptotic indices of ovarian follicles in female wild boars. The experiment was conducted on 40 wild boars inhabiting north-eastern Poland. The effect of seasonal variations in the quantity and quality of ingested food on the concentrations of Fusarium mycotoxins and their metabolites in the blood of wild boars was analyzed. The observed differences in toxin levels were accompanied by changes in proliferation and apoptotic indices. Proliferation processes were most intense in autumn-winter and were least advanced in winter-spring. The intensity of apoptotic processes was inversely correlated with proliferation.

Key words: wild boar, mycotoxicosis, reproductive system, Fusarium toxins

Introduction

Moldy feeds have been long known as etiological factors that contribute to various diseases in livestock. Mycotoxins produced by molds induce various effects that are not always toxic (Gajęcka et al. 2013), but which deviate from the norm in humans and animals (Morgavi and Rile 2007, De Saeger and van Egmond 2012). Filamentous fungi of the genus Fusarium are commonly found in feed ingredients and feeds. In nature, Fusarium fungi most often produce trace amounts of mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEN). Long-term exposure to mycotoxin doses below the no observable adverse effect level (NOAEL) causes pathological changes in accordance with the low-dose hypothesis (Vandenberg et al. 2012) and principles of hormesis (Frizell et al. 2011). The exposure to endocrine disruptors (Vandenberg et al. 2012), including ZEN, at dose levels

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below NOAEL may have stimulating/adaptive effects on pigs and wild boars (Heberer et al. 2007, Dobrzański and Fornalski 2011). Zearalenone, deoxynivalenol and mixed mycotoxicoses generally result from chronic subclinical infections characterized by weakly manifested and non-specific symptoms, which significantly impairs diagnosis. It remains unknown whether this condition is caused by the mycotoxin/mycotoxins alone or whether it is exacerbated by other factors, including environmental agents, or a homeostatic imbalance in animals exposed to mycotoxins (overload, dysfunctions, stage of the productive or reproductive cycle, stage of somatic development, such as sexual maturation). In the authors' opinion, wild animals are the best model for investigating the above correlations because the intensity of environmental factors to which they are exposed (Keuling et al. 2009) is similar throughout the year, therefore, their immune systems undergo continuous adaptation. By contrast, domesticated animals are protected against adverse environmental influences. The domestic pig (*Sus scrofa domestica*) is a livestock species that is most sensitive to the presence of ZEN, its metabolites and DON in feed (Gajęcki et al. 2010). Domestic pigs are members of the *Suidae* family and descendants of the wild boar that had been domesticated several thousand years ago (Morgami and Rile 2007).

ZEN and its metabolites, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), are characterized by structural similarity to oestrogen, but unlike steroids, they are not derived from steranic structures. Endocrine disruptors (EDs) such as ZEN can affect the hormonal system and induce adverse effects via several mechanisms (Barton 2012, Frizzell et al. 2011) including: (i) by competing with endogenous oestrogens for binding sites for oestrogen receptors (ERs) or androgen receptors in female pigs, which can lead to changes in mRNA expression and protein synthesis and lower the effectiveness of endogenous steroids, (ii) by binding to the receptor without activating it – the presence of foreign substances on the receptor prevents the binding of natural hormones (antagonistic effect), (iii) by binding to transport proteins in the blood and decreasing the concentrations of natural hormones in the blood stream, (iv) by disrupting metabolic processes in the body, affecting synthesis/degradation and the release of natural hormones.

DON is a trichothecene mycotoxin that contaminates feed ingredients of plant origin. Long-term exposure to low doses of DON reduces body weight gains and lowers appetite. Similarly to other trichothecenes, DON inhibits protein synthesis (Shephard 2011). Contaminated feed may lead to gastrointestinal disorders such as vomiting, feed refusal and bloody diarrhoea. In animals, the most common symptoms of long-term exposure to DON include suppressed body weight gain, anorexia and variations in nutritional efficiency (Scientific Report of EFSA 2013).

The wild boar is an ecotone species (Keuling et al. 2009) that lives in transitional zones between forest habitats, arable fields and human settlements, which is why the feed consumed by wild boars is characterized by various degrees of contamination. This prompted us to investigate the stimulating effects of environmental mycotoxins on the reproductive system and selected ovarian cells in wild boars.

The objective of this study was to determine the effect of natural mixed mycotoxicosis on the proliferation and apoptotic indices of ovarian follicles in female wild boars.

Materials and Methods

Animals

Samples for laboratory analyses were obtained from female wild boars inhabiting north-eastern Poland. The animals were hunter-harvested in four seasons of the year (first quarter of the year (astronomical spring) – GI, second quarter of the year (astronomical summer) – GII, third quarter of the year (astronomical autumn) – GIII, fourth quarter of the year (astronomical winter) – GIV). A minimum of 5-6 animals were harvested during each season over a period of two years (a total of 40 female boars – 20 in 2011 and 20 in 2012).

Blood sampling and preparation for mycotoxin analysis

Blood samples for the determination of ZEN, α-ZEL, β-ZEL and DON concentrations were collected immediately after hunter-harvesting in each quarter of 2011 and 2012. The samples were immediately transferred to chilled centrifuge tubes containing heparin and were centrifuged at 3000 rpm for 20 minutes at 4°C. The resulting plasma was placed in 3 ml Eppendorf tubes and freeze-stored at -20°C until analysis.

Determination of blood plasma concentrations of ZEN, ZEN metabolites and DON

Plasma concentrations of ZEN, α-ZEL, β-ZEL and DON were determined in the Department of

Veterinary Prevention and Feed Hygiene by combined separation techniques with the use of immunoaffinity columns (Zearala-Test™ Zearalenone Testing System, G1012, VICAM, Watertown, USA and DON-Test™ DON Testing System, VICAM, Watertown, USA) and high-performance liquid chromatography/mass spectrometry (LC/MS) based on the method proposed by Gajęcka et al. (2013).

Statistical analysis

The plasma concentrations of ZEN and its metabolites (Fig. 1) in female boars were expressed in terms of mean values ($\bar{x}$) and standard deviation (SD) for each sample. Data were analyzed in the Statistica application (StatSoft Inc., USA). Mean values were compared by one-way ANOVA for repeated measures to account for the applied ZEA dose and its administration period. The equality of variances in the compared groups was tested by the Brown-Forsythe test. The significance of differences between groups was estimated by Tukey's HSD post-hoc test ($p< 0.05$ or $p<0.01$).

Material sampling and sample preparation for immunohistochemical analysis

Post-mortem samples of the ovaries (left ovary) were collected immediately after hunter-harvesting. Samples were prepared and processed at the Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. Sections sampled for histopathological analyses were fixed in 10% formalin, neutralised and buffered to pH 7.4, and embedded in paraffin blocks. Microtome sections were stained with haematoxylin and eosin (HE) and PAS according to the method proposed by McManus (1999). Microscopic images ovarian cross-sections were analyzed in minimum of 20 fields of view under a light microscope (100 watt halogen lighting was used in the Olympus BX50 microscope) at 400x magnification.

PCNA analysis

The presence of the proliferating cell nuclear antigen (PCNA) was determined immunohistochemically with anti-PCNA mouse monoclonal antibodies (DAKO, clone PC-10, IgG2 kappa) diluted 1:150. Immunohistochemical reactions were identical for all specimens. The same reagents, time, temperature, moisture conditions were applied in all stains. Staining was performed on the same day by three collaborating observers. The staining procedure was performed with the use of the DAKO ARK™ Animal Research Kit for immunohistochemical staining of paraffin sections with mouse primary antibodies.

The immunoperoxidase staining method was used with the streptavidin-biotin-peroxidase complex solution to eliminate non-specific reactions. Following paraffin removal and hydration, the prepared material for PCNA analysis was placed in a citrate buffer with the pH of 6.0 and boiled twice in a 650 MW microwave oven for 5 min each to expose the antigens. To inactivate endogenous peroxidise activity, the specimens were incubated for 5 min in the presence of a peroxidase blocker provided in the kit. Prior to staining, anti-PCNA antibodies were mixed separately with biotinylated anti-mouse immunoglobulin (secondary antibody) at the calculated rates. The calculations for every preparation were performed independently by three observers, and the results were averaged. A biotinylated secondary antibody conjugate against the primary antibody was obtained. A mouse serum blocking reagent was added to fix the biotinylated anti-mouse immunoglobulin that was not bound in previous reactions to minimize potential interactions with endogenous tissue immunoglobulin. The resulting biotinylated primary antibodies were added to the samples and incubated for 15±5 min. The slides were covered with peroxidase-labelled streptavidin, followed by 3,3'-diaminobenzidine as the chromogen to obtain a stained reaction product. The specimens were additionally stained with hematoxylin, dried and fixed in Canadian balm. Negative controls were IgG2 kappa antibodies, and positive controls were commercially available sections (DAKO) (Baserga 1991, Bravo et al. 1981). The specimens stained by the McManus (1999) method (PAS) were viewed under 480x magnification to determine the number of cells that were stained highly intensely by the chromogen (PCNA+++), the number of intensely stained cells (PCNA++), the number of cells containing the antigen (PCNA+) and the number of cells without the antigen (PCNA-).

The results were processed statistically, and the PI of ovarian structures of the analysed female wild boars are presented in tabular form (Fig. 2). The PI is a percentage value calculated as the ratio of PCNA+, PCNA++ and PCNA+++ cells to total PCNA- cells in each structure (Sobańska et al. 2007, Falco 2009). The applied method involved the calculation of 1000 cells in random fields under 100x magnification, and cell reactions were described as (+) or (-).

TUNEL test

The induction of apoptosis in cultured granulosa cells was investigated with the ApoAlert DNA Fragmentation Assay Kit (Clontech). Tissue sections were deparaffinised in xylene and rehydrated by graduated ethanol washes. The sections were cultured with the analyzed reagents for 48 h, the medium was removed, and the cells were rinsed twice with 200 µl of PBS (pH 7.4, room temperature). The cells were immersed in 4% formaldehyde, fixed for 25 minutes and rinsed twice with PBS. To permeabilize cell membranes, the cells were incubated with 0.2% Triton X-100 (6 min, on ice) and rinsed with PBS (5 min, room temperature). The growth chamber was removed from the slide, and the cells were covered with equilibration buffer (100 µl/well). A cover glass was placed on the slide to ensure even distribution of the buffer. The specimens were equilibrated for 10 min. An incubation buffer containing terminal eoxynucleotidyl transferase was prepared. The buffer for every reaction comprised: 45 µl of equilibration buffer, 5 µl of the nucleotide mix and 1 µl of terminal deoxynucleotidyl transferase. After 10 min of equilibration, the cover glass was removed and excess buffer was drained off. The cells were covered with the incubation buffer (50 µl/well), and a cover glass was placed on the slide. The specimens were incubated in darkness (at 37°C, on a Petri dish lined with wet filter paper, 75 min). After incubation with terminal deoxynucleotidyl transferase, the cells were covered with 2x SSC buffer to inhibit enzyme activity (15 min, room temperature, in the dark). The slides were rinsed with PBS (5 min, room temperature), and propidium iodide was applied (propidium iodide/PBS, 10 µg/ml, 6 min, room temperature, in the dark). To remove excess staining solution, the specimens were rinsed three times with redistilled water and incubated for 5 min at room temperature. Every experiment was performed in four replications. The cells were observed under a fluorescence microscope (520 nm wavelength) directly after the last rinsing. Six TUNEL images were obtained for every specimen, one for each factor studied. The total number of granulosa cells and the number of apoptotic cells were counted in each image. The results were presented as the apoptotic index (AI) indicating the percentage of apoptotic cells. The results were arcsine transformed before statistical analysis. To verify the quality of the TUNEL assay, the cells were treated with DNase as positive control (two doses of 25 U/ml and 50 U/ml). To produce a positive biological control, the cells were cultured on a medium containing popular apoptosis inducers: tumour necrosis factor-α (10 ng/ml, 100 ng/ml, 1 µg/ml), staurosporine (0.1 nM, 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM) and actinomycin D (act. D: 500 ng/ml, 1 µg/ml). Negative control comprised cells incubated in buffer without terminal deoxynucleotidyl transferase. Three independent observers evaluated apoptotic cells. The number of apoptotic cells was determined in 10 fields of view covering a total of around 1000 cells, and the number of cells in each field was summed to produce the AI. The results were averaged when the AI values given by independent observers differed (Fig. 3).

Statistical analysis

PI and AI values are presented as arithmetic means $\bar{x}$ and SD of the mean (Fig. 2, 3). Data were processed in the Statistica application (StatSoft Inc., USA). The resulting means were compared by one-way ANOVA for repeated measures and Fisher's LSD post-hoc test ($p< 0.05$ or $p<0.01$).

Results

Mycotoxins analysis

Blood plasma concentrations of ZEN, α-ZEL and β-ZEL at different sampling dates are presented in Fig. 1. ZEN and its metabolites were present in all animal groups. DON values were below the sensitivity of the method and were within the range of analytical error.

The lowest concentrations of ZEN and its metabolites were found in GII, and this group was used as the reference in statistical analysis. The presence of the above toxins in group GII was determined only with the use of high-precision LC/MS equipment (Goyarts et al. 2010, Gajęcka et al. 2013). At $p<0.05$, differences in ZEN concentrations were reported between GII vs. GI and GIV, differences in α-ZEL levels were noted between GII vs. GI and GIII, and differences in β-ZEL concentrations – between GII and GIV. At $p<0.01$, differences in α-ZEL levels were observed between GII and GIV.

Immunohistochemical analysis

The results of PCNA expression and TUNEL reactions were processed statistically in immunohistochemical assays to obtain PI and AI values in numerical form. PI and AI values for selected ovarian structures of female wild boars are presented in Fig. 2 and 3.

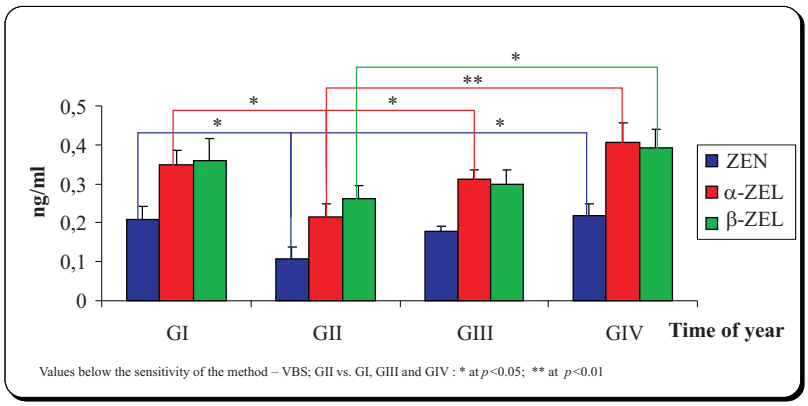

Fig. 1. Plasma concentrations of ZEN, α-ZEL and β-ZEL in female wild boars at different sampling dates ($\bar{x}$, SD)

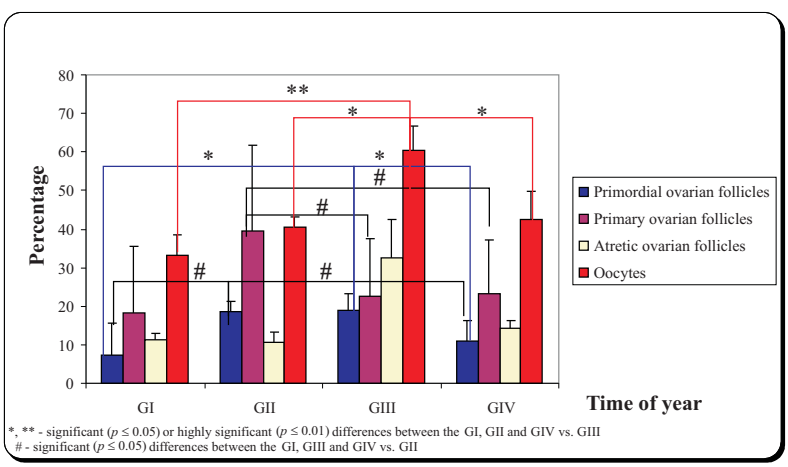

Fig. 2. Average proliferation index values in selected ovarian structures in female wild boars ($\bar{x}$, SD)

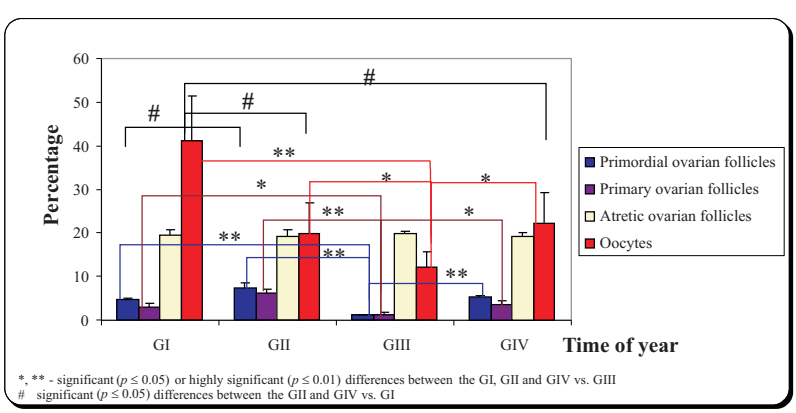

Fig. 3. Average apoptotic index values in selected ovarian structures in female wild boars ($\bar{x}$, SD)

The PI values for selected ovarian structures of female wild boars (Fig. 2) indicate that proliferation processes were most intense in GIII, but in all the tissues examined, the highest PI values were observed in oocytes in all groups. In oocytes, significant differences ($p \leq 0.05$) were found between GIII vs. GIV and GII (activity levels were higher by 18.07 PI in GIV and by 19.91 PI in GII), and highly significant differences ($p \leq 0.01$) were observed between GIII and GI (activity levels were lower by 27.33 PI in GI). In primordial ovarian follicles, significant differences were observed between GIII vs. GIV and GI (PI values were lower by 7.85 and 11.58, respectively), and between GII vs. GIV and GI (PI values were lower by 7.61 and 11.34, respectively). In primary ovarian follicles, significant differences were observed between

GII vs. GIV and GIII (PI values were lower by 16.09 and 16.90, respectively). In atretic ovarian follicles, significant differences were found between GIII vs. GI, GII and GIV (PI values were lower by 18.32, 21.32 and 21.92, respectively).

The average PI values (Fig. 2) were not normally distributed, and the median for all structures was determined at 25.33 PI with 7.38 PI in the lower quartile and 60.51 PI in the upper quartile. Subject to the value of the median and the lower and upper quartile, the results were divided into four activity levels: A – very low PI (PI < 15), B – low PI (15 ≤ PI ≥ 25), C – high PI (25 ≤ PI ≥ 35), D – very high PI (PI > 35). The lowest PI value corresponding to activity level A was observed in primordial follicles in GI. The highest PI value corresponding to activity level D was determined in oocytes in GIII. In general, the highest PI values were observed in oocytes (level D) in all experimental groups, in primary ovarian follicles (level D) in GII, and in atretic ovarian follicles (level C) in GIII. The lowest PI values were found in primordial ovarian follicles (level A) in groups GI and GIV, and in atretic ovarian follicles (level A) in GI, GII and GIV.

The apoptotic index (AI) was determined in 40 ovaries harvested from female wild boars. The results (Fig. 3) point to significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$) differences in the intensity of apoptosis in primordial ovarian follicles between GIII vs. GI, and GIV and between GI vs. GIV and GII. In the values noted in atretic ovarian follicles no significant differences were observed. In oocytes, significant differences were found between GIII vs. GIV and GII (AI values were higher by 10.30 and 7.81, respectively) and between GI vs. GIII and GII (AI values were lower by 18.92 and 21.41, respectively), whereas a highly significant difference was noted between GIII and GI (AI value was higher by 29.22). In primordial ovarian follicles, highly significant differences were observed between GIII and the remaining groups (AI values were higher by 4.13, 3.62 and 6.27, respectively). In primary ovarian follicles, significant differences were found between GIII vs. GIV and GI (AI values were higher by 2.25 and 1.81, respectively) and between GI and GII (AI value was lower by 3.12), whereas a highly significant difference was noted between GIII and GII (AI value was lower by 4.93).

AI values were not evenly distributed, and the median for all structures and groups was determined at 12.85 AI with 1.10 AI in the lower quartile and 41.23 AI in the upper quartile (Fig. 3). The median values for experimental groups reached: 12.57 AI for GIV, 17.14 AI for GI, 13.15 AI for GII and 8.55 AI for GIII. Subject to the value of the median for all groups and the lower and upper quartile, the results were divided into four activity levels: A – very low AI (AI < 5), B – low AI (5 ≤ AI ≥ 13), C – high AI (13 ≤ AI ≥ 21), D – very high AI (AI > 21). The lowest AI value corresponding to activity level A was observed in primordial and primary ovarian follicles in all groups, and the lowest AI values were noted in GIII. Low AI values corresponding to activity level B were observed only in oocytes in GIII. High AI values corresponding to activity level C were reported in oocytes in GII and in atretic ovarian follicles in all groups. The highest AI values (activity level D) were found in oocytes in GIV and GI.

Discussion

Due to lack of published studies concerning zearalenone mycotoxicosis in wild boars (*Sus scrofa*), the results obtained were compared with those dealing with zearalenone mycotoxicosis in domestic pigs (*Sus scrofa* f. *domestica*). Both species belong to the genus *Sus* and the family of *Suidae*.

Health problems in wild boars and zoonotic diseases transmitted by the species have to be analyzed in view of their specific habitats (Boadellaa et al. 2012). The wild boar is an ecotone species that lives in transitional zones between different ecosystems. Wild boar population continue to increase in Poland and in other countries. The above decreases in physical tolerance and competitiveness in forest ecosystems (van Ginkel et al. 2013, Liu et al. 2013) prompt boars to search for food in fields, meadows or human settlements (Keuling et al. 2008, 2010). Ecotones are narrow transitional zones between different ecosystems, they are characterized by labile boundaries and are commonly used as indicators of climate change (Wasson et al. 2013).

Estrogenic compounds are widely spread in the environment, and they may exert uncontrolled effects in all ecosystems inhabited by wild boars. Estrogens are found naturally in the body, and they also occur in the environment and in the form of xenobiotics. Environmental estrogens have been recently identified, some of them as undesirable compounds (Gajęcki et al. 2010), and they are classified as endocrine disruptors (EDs) (Yurino et al. 2004, Crain et al. 2008). Exposure to high levels (above NOAEL) of EDs can lead to pathological processes, in particular in the reproductive system, specially in the ovaries. Environmental EDs include mycoestrogens produced by molds, such as ZEN (Dunbar et al. 2012). ZEN blocks ERs or mimics steroid hormones, in particular estradiol (Brevini et al. 2005). Even at very low concentrations, ZEN and its metabolites stimulate cell proliferation sensitive tissues in various animal species, which is consistent with principles of hormesis (Frizell

et al. 2011, Gajęcka et al. 2011, Gajęcka 2013). At concentrations below NOAEL (threshold values), many toxic compounds, including undesirable substances such as ZEN, exert stimulating/adaptive effects (Heberer et al. 2007, Dobrzański and Fornalski 2011), but these effects are weaker than those in organisms not exposed to a given toxic substance. Our experiment did not involve a control group, but the PI values observed (Fig. 2) were significantly lower in all seasons of the year (PI was determined in the range of 7 in spring to nearly 40 in primary ovarian follicles and 60 in oocytes) in comparison with the values found in pigs with experimentally induced hyperestrogenism (Gajęcka et al. 2011).

The results reported in a study on pigs were significantly higher because the animals were exposed to relatively high ZEN doses of 20 and 40 µg ZEN/kg BW over a period of 48 days. In this study, wild boars were exposed to inhomogeneous doses (Vandenberg et al. 2012) of environmental toxins whose natural concentrations were probably considerably lower. By analogy to background ionizing radiation, this could be described aś background distribution of mycotoxins'. ZEN is present in plant material throughout the year, and its concentrations are highest in autumn and winter, which suggests that proliferation processes should be most strongly inhibited in these seasons. This is a very important consideration because even mild hyperestrogenism (total endogenous and exogenous steroids) leads to changes in the activity of steroidogenic enzymes or changes in hormonal regulation at the prereceptor level. In the wild boars investigated, hormone concentrations, in particular progesterone levels, probably increased insignificantly (proportionally to the ZEN dose), which is an important consideration during pregnancy in the autumn/winter season.

The above assumptions are validated by the seasonality of reproduction in wild boars. Seasonal reproduction patterns are probably determined by the number of daylight hours and food availability. Both factors affect the reproductive status of wild boars (Kozdrowski and Dubiel 2004). The uptake of undesirable substances, such as ZEN, with plant material is also an important consideration. ZEN exposure increases with food uptake. For this reason, optimal environmental conditions and optimal hormonal status for reproduction are observed in autumn/winter. Female boars that conceive during this season give birth in a period that is most supportive for the mother and the offspring. The above is validated by the highest PI values (Fig. 2) and the lowest AI values in autumn. Oocytes are stimulated, which is important for future reproduction. The drop in apoptotic activity is probably related to an improvement in the animals' overall condition and a higher number of mature eggs released during ovulation in the mating season. A directly proportional correlation is observed between the animals' condition and their reproductive ability (Kozdrowski and Dubiel 2004), and ZEN probably plays an active role in this process.

In this study, peripheral blood concentrations of ZEN and its metabolites were very low, but significant differences were found in all seasons of the year relative to the values reported in summer (Fig. 1). In summer, food is readily available in forest ecosystems, and boars do not have to search for plant material in meadows, fields or human settlements, i.e. habitats that are most highly contaminated with molds. The highest levels of ZEN and its metabolites were observed in winter, and the end of winter marks the time of farrowing in wild boars. At that time, the proliferation process slows down and apoptosis is enhanced, i.e. there is a negative correlation between ZEN concentrations and ovulatory activity because anoestrus in female boars is generally observed in spring and summer (Fonseca et al. 2011). After farrowing, mothers have to feed their offspring at a time when food is scarce, at least in the initial period.

The proapoptotic effect of ZEA on ovarian structures overlaps the natural physiological mechanism, which is not the only possible cause of retrograde changes. There is a growing body of evidence to suggest that apoptosis and necrosis could be the final stages of the same process, at least in some cases. A study of equine ovarian follicles demonstrated that the choice between apoptosis and necrosis during follicular atresia is determined by the amount of energy stored in an egg cell (Alonso-Pozos et al. 2003). Unlike necrosis, apoptosis is an active process that requires energy, and when ATP reserves run out, the cell becomes necrotized. Food shortages (hunger) may be observed in late winter and early spring.

The presence of pathogens in ingested food can modulate the local immune response. The degree of immunomodulation is determined by the dose of the pathogenic compound, such as mycotoxins or ZEN. Clinical intoxication levels are not proportional to the ingested dose, which is consistent with the low-dose hypothesis proposed by Vandenberg et al. (2012). The induction of regulatory T cells (Tregs) is one of the mechanisms used by pathogens to escape the immune response (Silva-Campa et al. 2012). The above is observed during chronic or permanent infections of low intensity, and it could be regarded as a form of nutritional adaptation.

The effects of mycotoxins produced by molds, which are ingested with plant material in small quantities over long periods of time, remain largely unknown. According to unpublished studies, mycotoxins

ingested in very small doses escape the local immune response, and the above could considerably affect nutritional adaptation.

It can be concluded that wild boars inhabiting ecotone zones between forest, field, meadow and suburban habitats are exposed to low ZEN doses that could exert a minor regulatory effect on steroidogenesis, proliferation and apoptosis in the ovaries of female boars, subject to season. Proliferation processes were most intense in autumn-winter and were least advanced in winter-spring. The intensity of apoptotic processes was inversely correlated with proliferation.

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References

Alonso-Pozos I, Rosales-Torres AM, Avalos-Rodriguez A, Vergara-Onofre M, Rosado-Garcia A (**2003**) Mechanism of granulosa cell death during follicular atresia depends on follicular size. Theriogenology 60: 1071-1081.

Barton M (**2012**) Position paper: The membrane estrogen receptor GPER – Clues and questions. Steroids 77: 935-942.

Baserga R (**1991**) Growth regulation of the PCNA gene. J Cell Sci 98: 433-436.

Boadella M, Barasona JA, Pozio E, Montoro V, Vicente J, Gortazar C, Acevedo P (**2012**) Spatio-temporal trends and risk factors for *Trichinella* species infection in wild boar (*Sus scrofa*) populations of central Spain: a long-term study. Int J Parasitol 42: 739-745.

Bravo R, Frank R, Blundell PA, Mac Donald-Bravo H (**1981**) Cyclin/PCNA is the auxiliary proteins of DNA polymerase- delta. Nature 326: 515-517.

Brevini TA, Zanetto SB, Cillo F (**2005**) Effects of endocrine disruptors on developmental and reproductive functions. Curr Drug Targets Immune Endocr Metabol Disord 5: 1-10.

Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho S, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, Swan S, Walker C, Woodruff TK, Woodruff TJ, Giudice LC, Guillette LJ Jr (**2008**) Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 90: 911-940.

De Saeger S, van Egmond HP (**2012**) Special issue: masked mycotoxins. World Mycotoxin J 5: 203-206.

Dobrzyński L, Fornalski KW (**2011**) Hormesis – natural phenomenon of answer of organism on stress. Proceedings, of VII International Scientific Conference: *Veterinary Feed Hygiene – The Effects of Mycotoxins on Gastrointestinal Function.* 23-24 September 2011, Olsztyn, Poland, pp 6-14.

Dunbar B, Patel M, Fahey J, Wira C (**2012**) Endocrine control of mucosal immunity in the female reproductive tract: impact of environmental disruptors. Mol Cell Endocrinol 354: 85-93.

Falco M (**2009**) Proliferation index and its prognostic value in invasive ductal breast carcinoma. Ann Acad Med Stetin 55: 22-30.

Fonseca C, Alves da Silva A, Alves J, Vingada J, Soares AM (**2011**) Reproductive performance of wild boar females in Portugal. Eur J Wildl Res 57: 363-371.

Frizzell C, Ndossi D, Verhaegen S, Dahl E, Eriksen G, Sørlie M, Ropstad E, Muller M, Elliott CT, Connolly L (**2011**) Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. Toxicol Lett 206: 210-217.

Gajęcka M (**2013**) The effect of experimental low zearalenone intoxication on ovarian follicles in pre-pubertal bitches. Pol J Vet Sci 16: 45-54.

Gajęcka M, Rybarczyk L, Zwierzchowski W, Jakimiuk E, Zielonka Ł, Obremski K, Gajęcki M (**2011**) The effect of experimental, long-term exposure to low-dose zearalenone mycotoxicosis on the histological condition of ovaries in sexually immature gilts. Theriogenology 75: 1085-1094.

Gajęcka M, Zielonka Ł, Dąbrowski M, Mróz M, Gajęcki M (**2013**) The effect of low doses of zearalenone and its metabolites on progesterone and 17β-estradiol concentrations in blood of pre-pubertal female beagle dogs. Toxicon 76: 260-269.

Gajęcki M, Gajęcka M, Jakimiuk E, Zielonka Ł, Obremski K (**2010**) Zearalenone – undesirable substance. In: Rai M, Varma A (eds) Mycotoxins in food, feed and bioweapons. Springer-Verlag, Berlin Heidelberg, pp 131-144.

Goyarts T, Brüssow KP, Valenta H, Tiemann U, Jäger K, Dänicke S (**2010**) On the effects of the *Fusarium* toxin deoxynivalenol (DON) administered *per os* or intraperitoneal infusion to sows during days 63 to 70 of gestation. Mycotoxin Res 26: 119-131.

Heberer T, Lahrssen-Wiederholt M, Schafft H, Abraham K, Pzyrembel H, Henning KJ, Schauzu M, Braeunig J, Goetz M, Niemann L, Gundert-Remy U, Luch A, Appel B, Banasiak U, Böl GF, Lampen A, Wittkowski R, Hensel A (**2007**) Zero tolerances in food and animal feed-are there any scientific alternatives? A European point of view on an international controversy. Toxicol Lett 175: 118-135.

Keuling O, Lauterbach K, Stier N, Roth M (**2010**) Hunter feedback of individually marked wild boar Sus scrofa L.: dispersal and efficiency of hunting in northeastern Germany. Eur J Wildl Res 56: 159-167.

Keuling O, Stier N, Roth M (**2009**) Commuting, shifting or remaining? Different spatial utilisation patterns of wild boar *Sus scrofa* L. in forest and field crops during summer. Mamm Biol 74: 145-152.

Keuling O, Stier N, Roth M (**2008**) Annual and seasonal space use of different age classes of female wild boar *Sus scrofa* L. Eur J Wildl Res 54: 403-412.

Kozdrowski R, Dubiel A (**2004**) The effect of season on the properties of wild boar (*Sus scrofa* L.) semen. Anim Reprod Sci 80: 281-289.

Liu T, Zhang S, Tang J, Li T (**2013**) Comparison and Analysis of Agricultural and Forest Land Changes in Typical Agricultural Regions of Northern Mid-latitudes. Chin Geogra Sci 23: 163-172.

McManus PM (**1999**) Frequency and severity of mastocytemia in dogs with and without mast cell tumors: 120 cases (1995-1997). J AmVet Med Assoc 215: 355-357.

Morgavi DP, Riley RT (**2007**) An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with *Fusarium* toxins. Anim Feed Sci Tech 137: 201-212.

Scientific Report of EFSA (**2013**) Deoxynivalenol in food and feed: occurrence and exposure. EFSA J 11(10): 3379

Shephard GS (**2011**) Fusarium mycotoxins and human health. Plant Breeding Seed Sci 64: 113-121.

Silva-Campa E, Mata-Haro V, Mateu E, Hernández J (**2012**) Porcine reproductive and respiratory syndrome virus induces $CD4^+CD8^+CD25^+Foxp3^+$ regulatory T cells (Tregs). Virology 430: 73-80.

Sobańska E, Bar JK, Gryboś M, Gabryś M (**2007**) Evaluation of prognostic value of cyclin D1, cyclin E, p27 protein and proliferative activity PCNA in ovarian carcinomas. Onkol Pol 10: 97-104.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR Jr, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, Zoeller RT, Myers JP (**2012**) Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev 33: 378-455.

van Ginkel HA, Kuijper DP, Churski M, Zub K, Szafrańska P, Smit C (**2013**) Safe for saplings not safe for seeds: Quercus robur recruitment in relation to coarse woody debris in Białowieża Primeval Forest, Poland. Forest Ecol Manag 304: 73-79.

Wasson K, Woolfolk A, Fresquez C (**2013**) Ecotones as Indicators of Changing Environmental Conditions: Rapid Migration of Salt Marsh-Upland Boundaries. Estuar Coast 36: 654-664.

Yurino H, Ishikawa S, Sato T, Akadegawa K, Ito T, Ueha S, Inadera H, Matsushima K (**2004**) Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. Toxicol Sci 81: 139-147.