

Effects of Zearalenone, α -Zearalenol, and Genistein on Boar Sperm Motility *In Vitro*

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ABSTRACT

Krejčárková A., Folková P., Šimoník O., Šásková M., Krejčířová R., Drábek O., Rajmon R. (2017): **Effects of zearalenone, α -zearalenol, and genistein on boar sperm motility *in vitro*.** Czech J. Anim. Sci., 62, 435–445.

Genistein (GEN) and zearalenone (ZEA), environmental oestrogens commonly present in feedstuff for pigs, are known for their effects on reproductive functions. The aim was to verify the *in vitro* effects of 0.5–20 μ M concentrations of GEN, ZEA and its metabolite α -zearalenol (α -ZOL) on pig sperm motility. A dose-dependent increase of the immotile sperm amount against fast and medium-fast sperm clusters was observed with all three oestrogens from the lowest concentrations tested. Individual CASA (computer-assisted sperm analysis) parameters of motile sperms seemed to be less sensitive indicators. This should be considered especially in toxicological research on a sperm model. Background of inconsistencies in to date-published papers is discussed. The results shift the effective concentrations of ZEA, α -ZOL, and GEN to values achievable *in vivo* and raises the questions of risk assessment of these compounds in pig reproduction.

Keywords: pig; spermatozoa; environmental oestrogens; motility; CASA; cluster analysis

The environment commonly comprises substances that can affect hormonal homeostasis in an organism thanks to their ability to bind on endogenous receptors, so-called endocrine disruptors (Cederroth et al. 2010). Mycotoxin zearalenone (ZEA) and its metabolites or soy phytoestrogens, mainly aglycones genistein (GEN) and daidzein (DAI), belong among these substances; despite their non-steroidal structure they can bind to the oestrogen receptors in mammalian cells (Majdic 2010). Given the popularity of soybean meal in the current pig nutrition and the high sensitivity of pigs (Benzoni et al. 2008), the question regarding

the effects of these substances on pig reproduction is still relevant.

Boar exposition to ZEA resulted repeatedly in generally negative effects on their reproductive system (Benzoni et al. 2008; Kanora and Maes 2009; Sutkeviciene et al. 2009; Bhat et al. 2010), whereas GEN had rather ambiguous effects (Yuan et al. 2012). Surprisingly, the semen quality was a rather peripheral interest in these *in vivo* experiments. Lower sperm motility was recorded after long-term feeding of ZEA at low concentrations (Young and King 1986). On the contrary, 250 ppm of GEN in feed stimulated the fructose level in

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (S grant) and by the Internal Grant Agency of the University of Life Sciences Prague (CIGA) (Project No. 20162027).

semen compared to the control group, resulting in its better quality (Yuan et al. 2012).

Under *in vitro* conditions, the inhibitory effects on the boar sperm motility were recorded for ZEA as well as for its major metabolite alpha-zearalenol (α -ZOL) at quite high concentrations of 125–250 μ M (Tsakmakidis et al. 2006, 2007). Lower concentrations seemed to be without any substantial effect (Benzoni et al. 2008; Tsakmakidis et al. 2008; Sambuu et al. 2013). On the other hand, after incubation with 31.2 μ M of α -ZOL, Gray et al. (2016) observed a reduction in the percentage of total sperm motility, percentage of rapid motility, percentage of progressive motility, and CASA (computer-assisted sperm analysis) parameters, such as VCL (curvilinear velocity), VSL (straight-line velocity), VAP (velocity on average path), ALH (amplitude of lateral head displacement), and LIN (linearity).

The effects of GEN on boar sperm motility were also ambiguous. Whereas Oh et al. (2011) recorded a reduction in total motility in the 0.001–100 μ M concentration range, Kim et al. (2014) did not observe any differences in the presence of 1, 50, and 100 μ M GEN compared to the control group, and furthermore, 50 μ M GEN even had stimulatory effects.

It is obvious that the reported effects of ZEA, α -ZOL, and GEN differ among studies. Furthermore, due to the differences in the used methodologies they are not easy to compare. There are differences in treatment conditions as well as in the evaluation of sperm motility. Some studies used subjective observation only (Tsakmakidis et al. 2008; Kim et al. 2014). Moreover, the approach to the objective assessment of sperm motility by CASA has changed over recent years (Simonik et al. 2015).

The aim of our study was to objectively assess the effects of these commonly available environmental oestrogens on the motility of boar ejaculated spermatozoa in the range of concentration where the minimum effect of these substances can be hypothesized, and using up-to-date sperm motility assessment methods, to elucidate the possible reasons for inconsistent results from published papers.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from Sigma Aldrich (USA) unless specified otherwise.

Stock solutions (10 mM) of ZEA (Z2125), α -ZOL (Z0166) or GEN (G6776) were prepared by dis-

solving lyophilized powder in dimethyl sulfoxide (DMSO; D5879) and stored at -20°C . Subsequently, working solutions were prepared every day using the sperm extender (SUS; Medichimica, Italy) as diluent.

Animals and sample collection. Semen of five boars with verified fertility (insemination station ProAgro Nymburk, Czech Republic) was used for experiments. Proven sperm-rich fractions with at least 75% of motile spermatozoa were extended immediately after collection by a sperm extender (SUS; Medichimica) to $15\text{--}20 \times 10^6$ spermatozoa per ml, transported and stored subsequently in standard insemination dose tubes at 17°C for further processing and analyses. Experiments were executed in the laboratory of the Department of Veterinary Science, Czech University of Life Sciences Prague no later than 48 h after collection. Before each experiment, sperm motility was checked again and only semen with $> 75\%$ of motile spermatozoa (subjective evaluation) at that time were used.

Experimental design. Three single experiments, each with oestrogen (ZEA, α -ZOL, and GEN) were carried out separately by the same operating procedure: aliquots (950 μ l) of semen were rewarmed in plastic Eppendorf microtubes to 38°C in a water bath (SUB 6; Grant Instruments, UK). Then 50 μ l of ZEA, α -ZOL or GEN solution in DMSO with extender were added to each microtube to reach final concentrations of the particular oestrogen as follows: 0 μ M (control), 0.5 μ M, 1 μ M, 2.5 μ M, 5 μ M, 10 μ M, and 20 μ M. The content of DMSO per sample was 2 μ l. All semen aliquots were then incubated under the same conditions (38°C in a water bath) and the sperm motility was examined after 2 and 4 hours of incubation. All tools and laboratory equipment used during the experiment were heated at 38°C . The experiment for each oestrogen was repeated at least six times.

Sperm motility analysis. The sperm motility was evaluated by the CASA module (Nis Elements, Version 3.20; Laboratory Imaging, Czech Republic). More specifically, 2 μ l of the sample were placed into a 20 μ M deep chamber (Leja[®] glass, the Netherlands) and the sperm motility was captured at six fields per chamber by a microscope with a heating table (Eclipse E600 (Nikon Corp., Japan), magnification $\times 100$, negative phase contrast; objective Ph 1 BM (Nikon Corp.); a camera ProgRes[®] CT1 (Jenoptik, Germany)). Sperm movement was recorded in a 2-s time lap with a frequency of 34.5

doi: 10.17221/19/2017-CJAS

frames/s, i.e. 69 digitalized images were processed. The track of each sperm was analyzed for the following kinematic parameters: curvilinear velocity (VCL ($\mu\text{M/s}$)), average-path velocity (VAP ($\mu\text{M/s}$)), straight-line velocity (VSL ($\mu\text{M/s}$)), amplitude of the lateral head displacement (ALH (μM)), beat cross frequency (BCF (Hz)), linearity (LIN; $\text{VSL/VCL} \times 100$ (%)), straightness (STR; $\text{VSL/VAP} \times 100$ (%)), and wobble (WOB; $\text{VAP/VCL} \times 100$ (%)).

Spermatozoa with $\text{VAP} > 10 \mu\text{M/s}$ were classified as motile. Median of the CASA kinematic parameters mentioned above was expressed from the population of motile spermatozoa, and the subpopulation structure was also analyzed. Finally, the percentage of IMMOTILE sperm ($\text{VAP} \leq 10 \mu\text{M/s}$) was expressed.

Statistical analysis. A statistical analysis was performed using STATISTICA software, Version 12 (StatSoft CR, Czech Republic). The following statistical tests were used: cluster analysis, chi-square test, ANOVA, Scheffé's test. With regard to the wide range of the analyzed files ($n > 6500$ in each experimental group) and the variability observed among semen aliquots before the treatment, the level of significance in all statistical tests was set at $P < 0.0001$.

To identify sperm subpopulations, a cluster analysis was performed. Motile spermatozoa CASA data sets from individual experiments were assessed by k-means clustering based on Euclidean distances of the chosen sperm motility descriptors (VCL, VSL, VAP, ALH, and BCF). Thus, motile spermatozoa were assigned to three specific disjointed clusters corresponding to three sperm subpopulations: SLOW, MEDIUM FAST, and FAST. For the purpose of detecting the sperm distribution in the individual subpopulation, a fourth category of IMMOTILE sperm ($\text{VAP} \leq 10 \mu\text{M/s}$) was added. Differences in the sperm distribution into subpopulations were checked using the chi-square test.

The effects of environmental oestrogens on the individual sperm kinematic parameters were evaluated by means of an analysis of variance (ANOVA) with interactions. Then the value of mean \pm SEM was expressed. Any statistically significant differences among the obtained values were consequently specified by the Scheffé's test.

RESULTS AND DISCUSSION

The aim of this study was to objectively assess the effects of three naturally occurring oestrogenic

compounds (ZEA, α -ZOL, GEN) on the motion activity of boar ejaculated spermatozoa. Sperm motility is widely used as a basic marker of semen or spermatozoa quality (Dzyuba et al. 2015; Zhang et al. 2015; Petelak and Krylov 2016; Simonik et al. 2016). Previous studies focusing on the effects of the above-mentioned substances differ in their conclusions as well as in their methodological approaches. But the method of motility assessment may significantly influence obtained results (Broekhuijsen et al. 2011). We applied objective motility evaluation by means of computer-assisted sperm analysis (CASA), using the cluster analysis method, which is suitable for resolving heterogeneity of the sperm motility data in discrete subpopulations (Martinez-Pastor et al. 2011). This was combined with the evaluation of the immotile sperm proportion and the individual CASA parameters.

The kinematic characteristics of motile sperm subpopulations (SLOW, MEDIUM FAST, FAST) in experiments on ZEA, α -ZOL, or GEN are shown in Tables 1, 2, and 3, respectively. A significant decrease in the mean values was observed between 2 and 4 hours of incubation for most of the parameters. Only the VCL and ALH parameters in α -ZOL and GEN experiments showed opposite trends in some subpopulations (Table 2 and 3). The IMMOTILE subpopulation was defined as sperm with a $\text{VAP} \leq 10 \mu\text{M/s}$ and these sperm were not included in the clustering procedure.

Evaluating the distribution of spermatozoa among subpopulations (IMMOTILE, SLOW, MEDIUM FAST, and FAST), significant differences were recorded as early as after 2 hours of incubation for all three compounds at all concentrations tested (Figures 1A, 2A, 3A). With the higher concentration of each compound, the percentage of sperms in subpopulations IMMOTILE (especially) or SLOW increased to the detriment of the FAST or MEDIUM FAST subpopulations generally. After a 4-hour incubation (Figures 1B, 2B, 3B) the effects were more pronounced. The effects can be ordered $\text{ZEA} < \alpha\text{-ZOL} < \text{GEN}$. Although the effects of these three oestrogenic compounds were tested separately, giving priority to testing the concentration series, which meant the comparison could only be made very approximately, this is – to our knowledge – the only paper evaluating the effects of these compounds on sperm under the same protocol.

Evaluating individual kinematic parameters, the efficacy of individual compounds has manifested

Table 1. Kinematic parameters characterizing motile boar sperm subpopulations in experiment on zearalenone effects (mean \pm SEM)

	Two-hour incubation			Four-hour incubation		
	SLOW	MEDIUM FAST	FAST	SLOW	MEDIUM FAST	FAST
VCL ($\mu\text{m/s}$)	49.06 \pm 0.18 ^a	99.25 \pm 0.13 ^a	144.45 \pm 0.18 ^a	43.43 \pm 0.13 ^b	83.14 \pm 0.13 ^b	133.59 \pm 0.30 ^b
VAP ($\mu\text{m/s}$)	26.01 \pm 0.10 ^a	61.33 \pm 0.10 ^a	87.46 \pm 0.13 ^a	20.66 \pm 0.06 ^b	44.47 \pm 0.10 ^b	72.81 \pm 0.19 ^b
VSL ($\mu\text{m/s}$)	21.22 \pm 0.11 ^a	54.64 \pm 0.12 ^a	78.58 \pm 0.16 ^a	17.02 \pm 0.07 ^b	38.81 \pm 0.12 ^b	63.99 \pm 0.23 ^b
ALH (μm)	4.44 \pm 0.03 ^a	6.51 \pm 0.02 ^a	8.80 \pm 0.02 ^a	3.78 \pm 0.02 ^b	5.48 \pm 0.02 ^b	7.88 \pm 0.03 ^b
LIN (%)	48.39 \pm 0.24 ^a	56.83 \pm 0.13 ^a	55.78 \pm 0.13 ^a	44.45 \pm 0.20 ^b	48.48 \pm 0.16 ^b	49.62 \pm 0.2 ^b
WOB (%)	57.30 \pm 0.20 ^a	63.23 \pm 0.11 ^a	61.70 \pm 0.11 ^a	52.10 \pm 0.17 ^b	54.99 \pm 0.13 ^b	55.99 \pm 0.17 ^b
STR (%)	78.66 \pm 0.24 ^a	88.28 \pm 0.12 ^a	89.38 \pm 0.11 ^a	80.44 \pm 0.21 ^b	85.84 \pm 0.16 ^b	86.87 \pm 0.18 ^b
BCF (Hz)	6.62 \pm 0.03 ^a	6.96 \pm 0.02 ^a	7.14 \pm 0.03 ^a	6.59 \pm 0.02 ^a	6.77 \pm 0.02 ^b	7.10 \pm 0.04 ^a

VCL = curvilinear velocity, VAP = velocity on average path, VSL = straight-line velocity, ALH = amplitude of lateral head displacement, LIN = linearity, WOB = wobble, STR = straightness, BCF = beat cross frequency

subpopulations were defined by cluster analysis of computer assisted semen analysis data sets (k-means clustering based on Euclidean distances); motile spermatozoa of all treatment (0, 0.5, 1, 2.5, 5, 10, and 20 μM of zearalenone) and incubation variants (2 or 4 hours) were involved

^{a,b}values with different superscripts in a row within the same subpopulation significantly differ between 2 and 4 hours of incubation ($P < 0.0001$, Scheffé's test)

rather differently (Figures 4–6). Similar courses of changes were seen for the velocity parameters (VCL, VAP, and VSL) and for the calculated parameters (LIN, STR, and WOB) and BCF as well. Therefore, the results for VCL, ALH, and LIN are presented only. For GEN data on BCF are also

shown. The trends differed among compounds. Values similar to the control with only a few exceptions characterize the ZEA experiment (Figure 4). Data on α -ZOL show a much wider variation – with significant deviations from the control, especially at concentrations above 2.5 μM (Figure 5). The

Table 2. Kinematic parameters characterizing motile boar sperm subpopulations in experiment on α -zearalenol effects (mean \pm SEM)

	Two-hour incubation			Four-hour incubation		
	SLOW	MEDIUM FAST	FAST	SLOW	MEDIUM FAST	FAST
VCL ($\mu\text{m/s}$)	48.75 \pm 0.10 ^a	91.49 \pm 0.10 ^a	141.76 \pm 0.19 ^a	43.52 \pm 0.13 ^b	87.18 \pm 0.17 ^b	144.43 \pm 0.34 ^b
VAP ($\mu\text{m/s}$)	23.41 \pm 0.06 ^a	47.69 \pm 0.07 ^a	69.94 \pm 0.14 ^a	19.09 \pm 0.06 ^b	40.90 \pm 0.13 ^b	63.01 \pm 0.24 ^b
VSL ($\mu\text{m/s}$)	19.18 \pm 0.06 ^a	40.55 \pm 0.09 ^a	58.33 \pm 0.18 ^a	14.27 \pm 0.08 ^b	33.45 \pm 0.15 ^b	49.99 \pm 0.30 ^b
ALH (μm)	4.00 \pm 0.02 ^a	5.82 \pm 0.02 ^a	8.20 \pm 0.02 ^a	4.74 \pm 0.03 ^b	5.77 \pm 0.03 ^a	7.89 \pm 0.04 ^b
LIN (%)	41.33 \pm 0.13 ^a	46.02 \pm 0.11 ^a	42.56 \pm 0.14 ^a	35.27 \pm 0.19 ^b	40.06 \pm 0.20 ^b	36.01 \pm 0.24 ^b
WOB (%)	49.91 \pm 0.11 ^a	53.48 \pm 0.09 ^a	50.51 \pm 0.12 ^a	47.02 \pm 0.16 ^b	48.32 \pm 0.17 ^b	44.80 \pm 0.20 ^b
STR (%)	80.04 \pm 0.15 ^a	83.79 \pm 0.12 ^a	81.96 \pm 0.15 ^a	72.56 \pm 0.25 ^b	79.31 \pm 0.24 ^b	77.09 \pm 0.31 ^b
BCF (Hz)	6.65 \pm 0.02 ^a	7.02 \pm 0.02 ^a	7.16 \pm 0.03 ^a	5.94 \pm 0.03 ^b	6.70 \pm 0.03 ^b	6.88 \pm 0.04 ^b

VCL = curvilinear velocity, VAP = velocity on average path, VSL = straight-line velocity, ALH = amplitude of lateral head displacement, LIN = linearity, WOB = wobble, STR = straightness, BCF = beat cross frequency

subpopulations were defined by cluster analysis of computer assisted semen analysis data sets (k-means clustering based on Euclidean distances); motile spermatozoa of all treatment (0, 0.5, 1, 2.5, 5, 10, and 20 μM of α -zearalenol) and incubation variants (2 or 4 hours) were involved

^{a,b}values with different superscript letters in a row within subpopulation significantly differ between 2 and 4 hours of incubation ($P < 0.0001$; Scheffé's test)

doi: 10.17221/19/2017-CJAS

Table 3. Kinematic parameters characterizing motile boar sperm subpopulations in experiment on genistein effects (mean \pm SEM)

	Two-hour incubation			Four-hour incubation		
	SLOW	MEDIUM FAST	FAST	SLOW	MEDIUM FAST	FAST
VCL ($\mu\text{m/s}$)	41.67 \pm 0.11 ^a	88.26 \pm 0.11 ^a	138.75 \pm 0.20 ^a	41.61 \pm 0.10 ^a	83.77 \pm 0.14 ^b	142.25 \pm 0.31 ^b
VAP ($\mu\text{m/s}$)	22.31 \pm 0.06 ^a	46.90 \pm 0.08 ^a	70.32 \pm 0.16 ^a	19.39 \pm 0.05 ^b	36.07 \pm 0.11 ^b	58.80 \pm 0.24 ^b
VSL ($\mu\text{m/s}$)	18.75 \pm 0.07 ^a	40.50 \pm 0.10 ^a	59.99 \pm 0.20 ^a	14.89 \pm 0.07 ^b	27.75 \pm 0.14 ^b	44.98 \pm 0.28 ^b
ALH (μm)	3.85 \pm 0.02 ^a	5.80 \pm 0.02 ^a	8.05 \pm 0.02 ^a	4.45 \pm 0.02 ^b	6.45 \pm 0.03 ^b	8.39 \pm 0.04 ^b
LIN (%)	52.54 \pm 0.2 ^a	47.97 \pm 0.13 ^a	44.93 \pm 0.16 ^a	41.35 \pm 0.20 ^b	35.13 \pm 0.19 ^b	33.10 \pm 0.23 ^b
WOB (%)	59.88 \pm 0.17 ^a	54.88 \pm 0.11 ^a	52.10 \pm 0.13 ^a	51.32 \pm 0.16 ^b	44.75 \pm 0.16 ^b	42.62 \pm 0.19 ^b
STR (%)	81.66 \pm 0.18 ^a	84.43 \pm 0.13 ^a	83.61 \pm 0.16 ^a	74.35 \pm 0.22 ^b	73.51 \pm 0.25 ^b	73.75 \pm 0.31 ^b
BCF (Hz)	6.66 \pm 0.02 ^a	7.08 \pm 0.02 ^a	7.32 \pm 0.03 ^a	6.1 \pm 0.02 ^b	6.33 \pm 0.03 ^b	6.60 \pm 0.04 ^b

VCL = curvilinear velocity, VAP = velocity on average path, VSL = straight-line velocity, ALH = amplitude of lateral head displacement, LIN = linearity, WOB = wobble, STR = straightness, BCF = beat cross frequency

subpopulations were defined by cluster analysis of computer assisted semen analysis data sets (k-means clustering based on Euclidean distances); motile spermatozoa of all treatment (0, 0.5, 1, 2.5, 5, 10, and 20 μM of genistein) and incubation variants (2 or 4 hours) were involved

^{a,b}values with different superscripts in a row within the same subpopulation significantly differ between 2 and 4 hours of incubation ($P < 0.0001$, Scheffé's test)

same concentration seems to be just as critical for the GEN effects (Figure 6).

Zearalenone is already known for its negative effects on the reproductive organs of boars from the dose of 100 ppm in feed (Kanora and Maes 2009). This corresponds to 10^1 – 10^3 ppb in tissues, i.e. around 1 μM (Prelusky 1994; own unpublished data), which is much less than effective concentrations (125 μM ZEA) reported from *in vitro* experiments on sperm motility by Tsakmakidis et al. (2006). However, our cluster analysis indicates

significant redistribution of spermatozoa among subpopulations even from the concentration of 0.5 μM (Figure 1). In addition, the mean values of some kinematic parameters after a 2-hour incubation indicate possible effects of this concentration, although the difference from the control is considered to be significant ($P < 0.0001$) at ALH only (Figure 4). Inhibitory effects of ZEA concentrations above 5 μM are clearly evident with both methods of sperm motility evaluation. All of these findings demonstrate that the effective ZEA

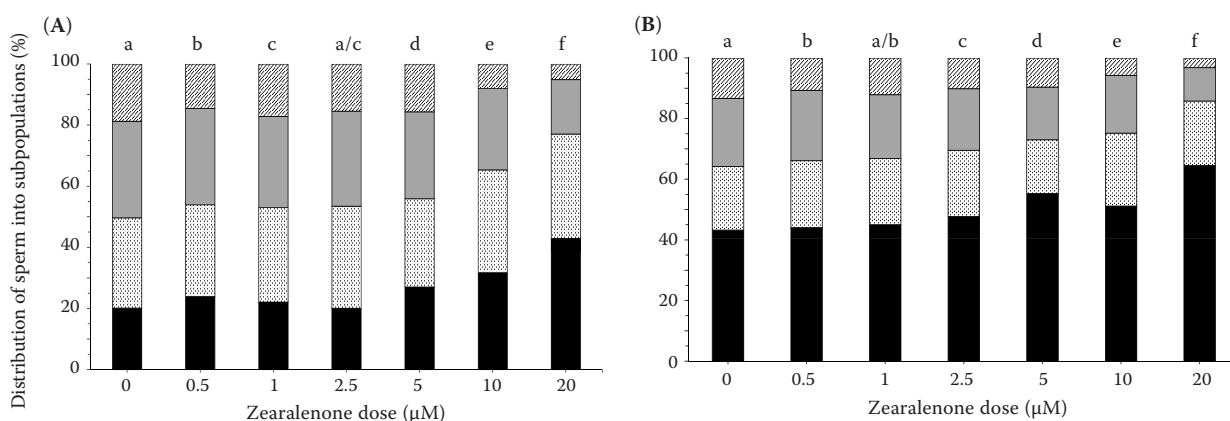


Figure 1. Percentage distribution of sperm into IMMOTILE (black bar), SLOW (spotted bar), MEDIUM FAST (grey bar), and FAST (striped bar) subpopulation after 2-hour (A) and 4-hour (B) incubation with different zearalenone doses ^{a–f}different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (chi-square test)

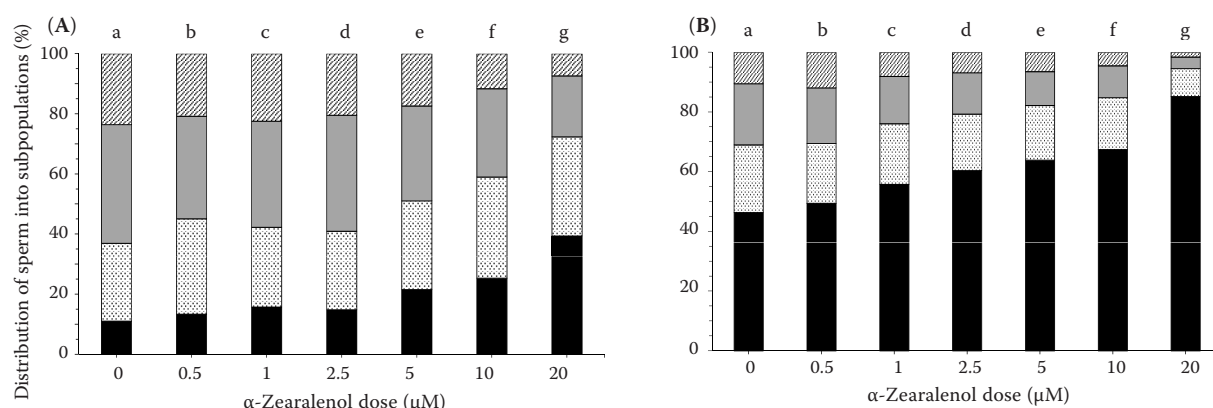


Figure 2. Percentage distribution of sperm into IMMOTILE (black bar), SLOW (spotted bar), MEDIUM FAST (grey bar), and FAST (striped bar) subpopulation after 2-hour (A) and 4-hour (B) incubation with different α -zearalenol doses a–g different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (chi-square test)

concentration on sperm motility is much lower than any yet published.

The alpha-zearalenol, resulting from the liver biotransformation of ZEA (Benzoni et al. 2008), is considered to be 10 times more potent than ZEA alone (Agag 2004). Tsakmakidis et al. (2008) did not observe any differences compared to the control group up to a concentration of 94 μM after 4 h of incubation. Nevertheless, Gray et al. (2016) recorded a negative influence of 31.2 μM of α -ZOL on sperm motility even after 30 min of incubation, and Benzoni et al. (2008) observed a decrease in VSL in boar spermatozoa under *in vitro* conditions at a concentration of 20 μM of α -ZOL. Our cluster analysis again indicates effects even from the lowest α -ZOL concentration tested, i.e. from

0.5 μM (Figure 2). Compared to ZEA, the changes are much more pronounced at the level of sperm subpopulations as well as with the evaluation of kinematic parameters (Figure 5) where significant deviations from the control were found after a 2-hour incubation with most of the concentrations, including the lowest one. This is in accordance with generally higher oestrogenic activity of α -ZOL (Fink-Gremmels and Malekinejad 2007).

Whereas ZEA and its metabolites are associated with rather negative effects on reproduction (Zinedine et al. 2007), phytoestrogens (such as GEN) can potentially act beneficially (Whitten and Patisaul 2001). There were observed no negative but even positive effects of GEN on boar semen under *in vivo* conditions (Yuan et al. 2012). Under

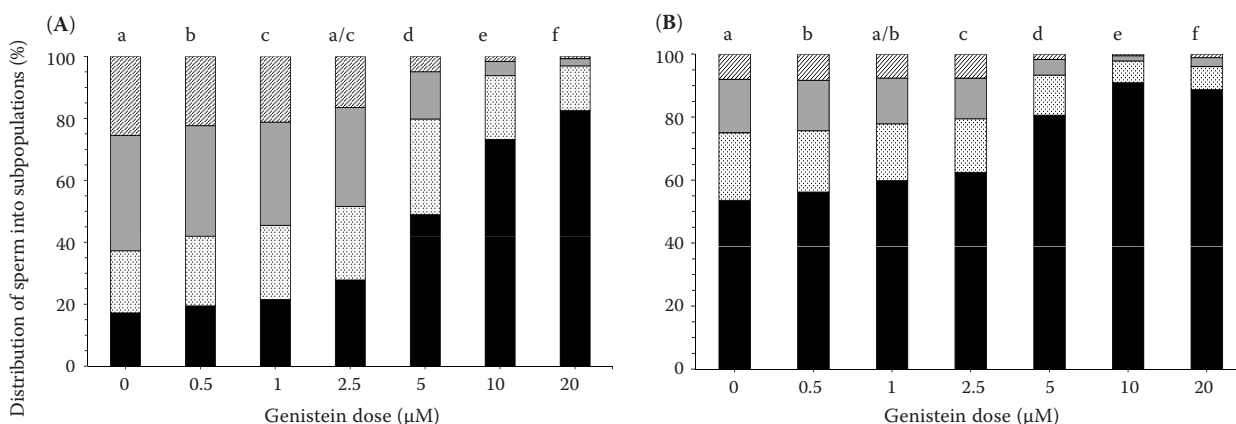


Figure 3. Percentage distribution of sperm into IMMOTILE (black bar), SLOW (spotted bar), MEDIUM FAST (grey bar), and FAST (striped bar) subpopulation after 2-hour (A) and 4-hour (B) incubation with different genistein doses a–g different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (chi-square test)

doi: 10.17221/19/2017-CJAS

in vitro conditions the reported GEN effects on boar semen are heterogeneous. Whereas Oh et al. (2011) recorded negative effects on sperm motility, Kim et al. (2014) observed no negative effects in most cases, or even some stimulation. Our data indicate similar changes to those seen with ZEA or α -ZOL – significant redistribution of sperms from the lowest tested concentration due to a decrease in the percentage of the two fastest subpopulations mainly in favour of immotile sperms. The effects seem to be even more pronounced – by cluster analysis (Figure 3) as well as individual kinematic parameter evaluation (Figure 6).

Comparing results of both analytic methods used, the cluster analysis seems more sensitive and also the most consistent. However, the combination of both methods can elucidate some of the non-compliance of our results with previously published work.

Although the ratio of sperm subpopulations exhibited a clear dependency on the ZEA dose as early as after a 2-hour incubation from the lowest ZEA concentration of 0.5 μ M, the velocity parameters of motile spermatozoa did not significantly differ from the control group in any of the tested concentrations. Moreover, the values for the lowest concentration of 0.5 μ M were even higher (for VCL significantly) compared to the other ZEA

concentrations (Figure 4A). The ALH curve looks very similar (Figure 4B). Obviously, the lowest ZEA concentration decreased the proportion of motile spermatozoa with a slight increase in the sperm movement activity. Oh et al. (2011) did not observe any effect on the individual CASA parameters in their genistein study either, but in most cases they detected an increase in the percentage of immotile spermatozoa, as we did in our case. It is, therefore, possible that although the ratio of immotile sperm increases, the characteristics of the so far motile spermatozoa do not change for a defined period. This can be caused by the fact that stress can evoke a transient stimulation of the sperm before its subsequent exhaustion. Given the heterogeneity of the sperm populations and the speed of their reaction to stress, this effect may be manifested as the absence of any impact on the average values of kinematic parameters or even as the increase in the average sperm velocity. This mechanism is indicated by increases in VCL mean values at the lowest ZEA concentration after a 2-hour incubation or in the FAST sperm cluster after a prolonged 4-hour cultivation (Table 1) – both situations were accompanied by the simultaneous losses of motile spermatozoa, or more precisely the losses of spermatozoa with the FAST and MEDIUM FAST motion. This relation-

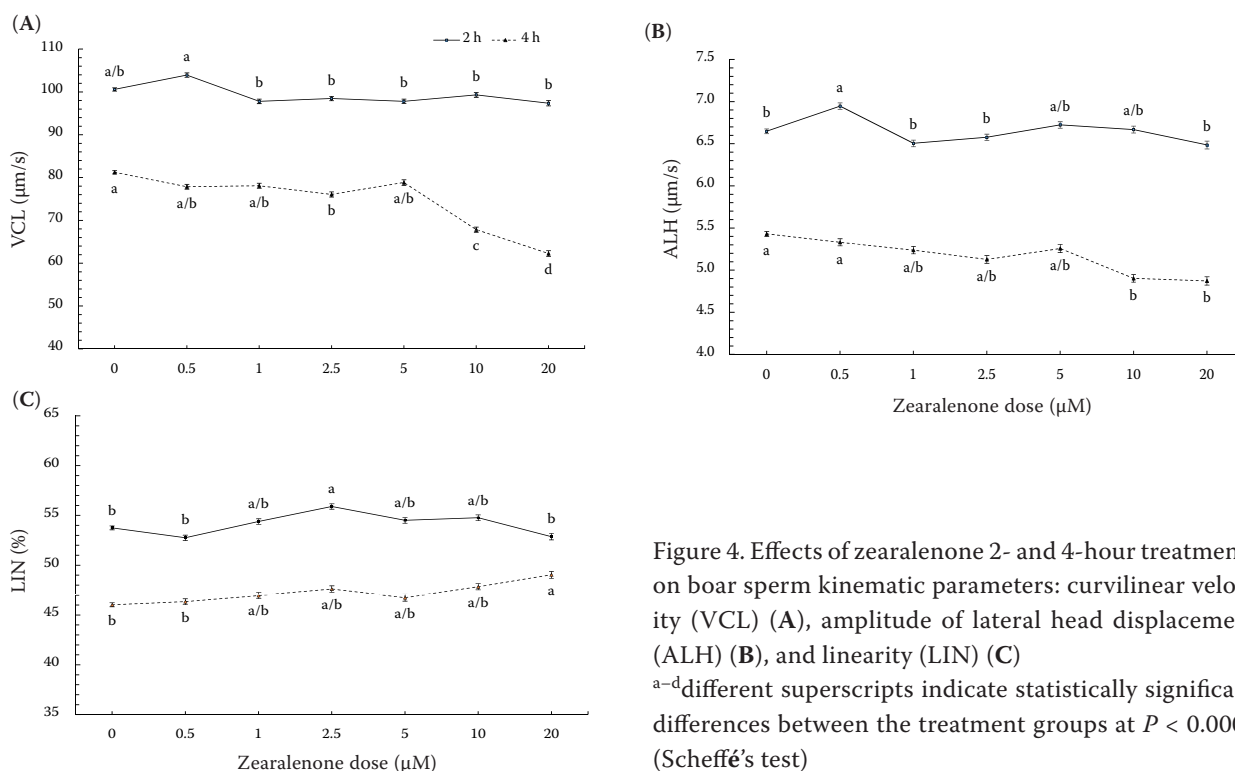


Figure 4. Effects of zearalenone 2- and 4-hour treatments on boar sperm kinematic parameters: curvilinear velocity (VCL) (A), amplitude of lateral head displacement (ALH) (B), and linearity (LIN) (C)

a–d different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (Scheffé's test)

ship is more visible in the VCL data as among the velocity parameters VCL can be more influenced due to its coinciding with function of the lateral head displacement (ALH).

Such behaviour of CASA data sets might explain the negative results of some previous papers. For example, Benzoni et al. (2008) also evaluated VCL and VSL in boar spermatozoa. After sperm exposition to ZEA (2 pM–20 μ M) they observed no effects of ZEA; however, authors did not provide the percentage of total motility.

Data on α -ZOL address another pitfall of kinematic parameters mean values. Contrary to the experiment with ZEA, a dose-dependent decrease in VCL values (Figure 5A) was found. The course of the curve for ALH (Figure 5B) mean values after a 2-hour incubation shows the same trend as for VCL. However, the findings after 4 hours of incubation look strange, because contrary to the decrease in VCL with increasing α -ZOL concentration, the ALH values were stable or (at the concentrations of 5 and 20 μ M) even increased. These abnormalities also appeared (in a mirror-like manner) with the BCF parameter and the parameters reflecting the straightness of motion

– LIN (Figure 5C), STR, WOB (data not shown). Thus, spermatozoa exhibited a higher deviation of lateral head displacement upon a slower frequency and larger curvature of their pathway. Mechanical assessment of individual parameters might lead to conclusions about the non-linear action of α -ZOL, stimulatory effects on ALH or progressivity of movement. However, comparing all available data it can be assumed that the mean values were actually affected by the presence of a higher amount of spermatozoa with a more or less local movement in the slow sperm category, which – with the overall small amount of motile sperm – markedly shifted the mean kinematic values. An increase in the ALH mean values characterizing the slow sperm cluster after 4 hours of incubation also corresponds with this suggestion. Similarly, a “surprising” increase of LIN parameter in GEN experiment is better understandable comparing data on sperm subpopulations – among low number of motile spermatozoa slow ones are predominant. As $LIN = VSL/VCL \times 100$ and boar spermatozoa show physiologically higher percentage of circulatory movement (Johnson et al. 2000), slow spermatozoa have higher LIN compared to fast ones even on the same trajectory.

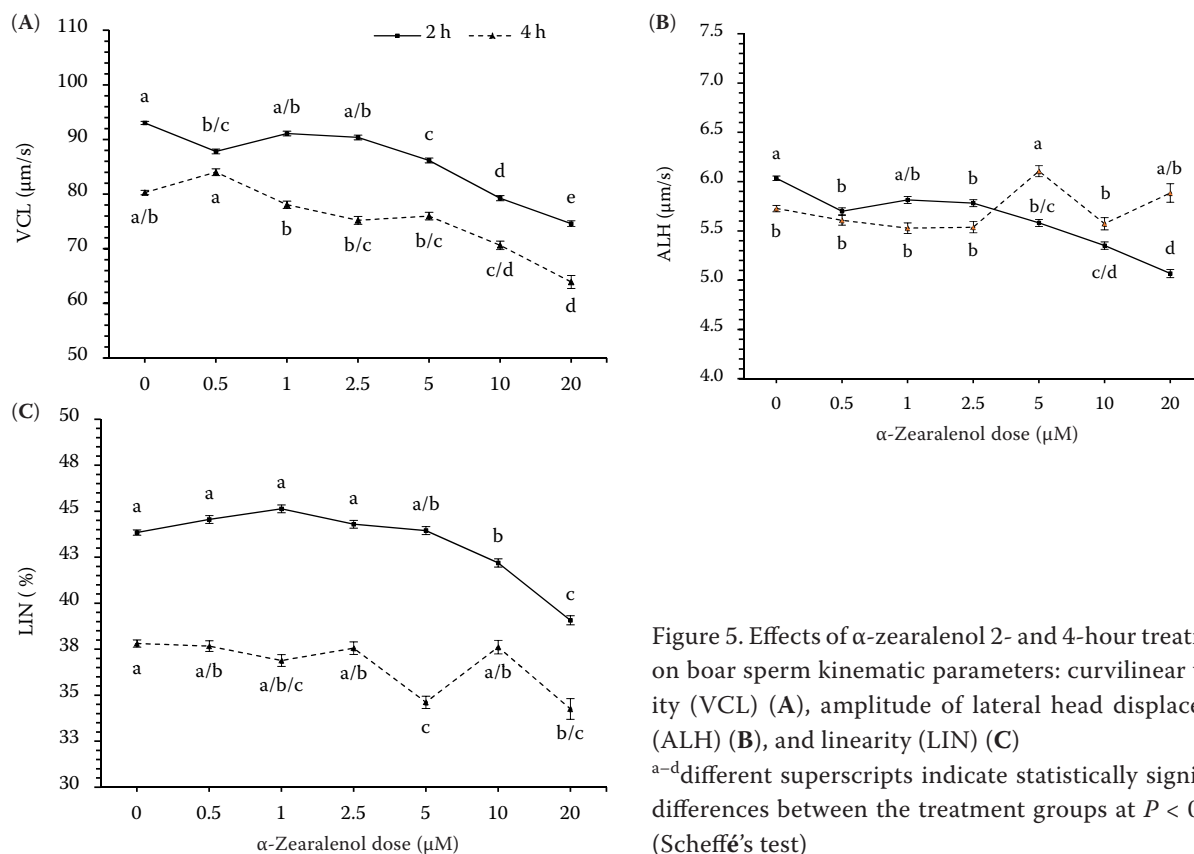


Figure 5. Effects of α -zearalenol 2- and 4-hour treatments on boar sperm kinematic parameters: curvilinear velocity (VCL) (A), amplitude of lateral head displacement (ALH) (B), and linearity (LIN) (C)

^{a–d}different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (Scheffé's test)

doi: 10.17221/19/2017-CJAS

It is obvious that the inhibition effects of GEN were most dominant among all three tested contaminants – e.g. 20 μM of GEN caused an increase in the immotile sperm ratio compared to the control group by about 65.3% (vs 28.34% for α -ZOL and 22.81% for ZEA). Also the dynamics of the mean value of kinematic parameters was clearly steeper. This distinct image can be related to slightly different impacts of GEN on metabolic pathways compared to ZEA and α -ZOL. Nevertheless, the oestrogenic activity of GEN is regarded as much lower than that of ZEA and its metabolites (e.g. Gromadzka et al. 2008). It is interesting that Oh et al. (2011) did not record any changes in the selected CASA parameters in the range of concentration from 0.001 to 100 μM of GEN, although they observed a significant increase in the number of immotile spermatozoa. As our experiments on individual oestrogenic compounds were performed one after the other in time, the ejaculate quality as a factor can remain in the background. It is evident that sperm resistance to the incubation conditions (see each sperm category in the control ratio, Figures 1–3) differed with every experiment. However, Oh et al. (2011) observed a decrease in the

total motility after GEN addition with a concentration of as little as 0.001 μM even after 15 or 30 min of incubation. Following on from the information mentioned above, no similar finding has yet been published for ZEA or α -ZOL. On the other hand, Kim et al. (2014) did not observe any effects on sperm motility at the same concentrations (1–100 μM of GEN) after 3 hours of incubation. This discrepancy might be due to the use of different evaluation methodologies – Kim et al. (2014) used subjective evaluation, Oh et al. (2011) the more objective CASA. But there may also be another explanation. GEN, as well as ZEA and its metabolites, are substances soluble in ethanol or DMSO, but very poorly soluble in water. Nevertheless, all procedures testing their effects on sperm are conducted in watery solutions, whereby these substances are dissolved in a polar solvent and then added to the watery sperm sample. Our experience (not published) shows that even small changes in the composition of the cultivation medium can lead to the precipitation of these substances or to their binding to other components which affect the availability of these substances and their effects. Nevertheless, none of the cited studies examined the

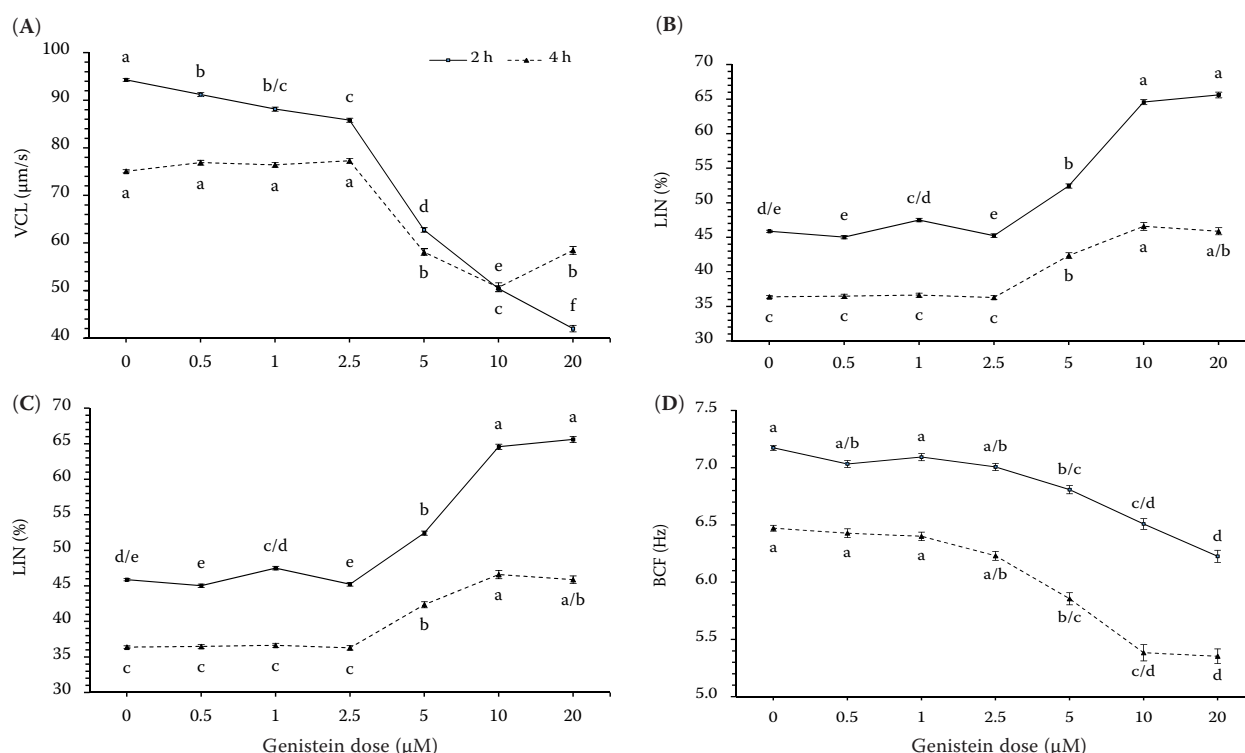


Figure 6. Effect of genistein 2- and 4-hour treatments on boar sperm kinematic parameters: curvilinear velocity (VCL) (A), amplitude of lateral head displacement (ALH) (B), linearity (LIN) (C), and beat cross frequency (BCF) (D) ^{a–f}different superscripts indicate statistically significant differences between the treatment groups at $P < 0.0001$ (Scheffé's test)

real concentration of contaminants in the medium. Therefore, it is necessary to take a cautious approach to the studies with negative findings.

The issue of availability of the effective substances may also underlie the not fully linear relationship of the observed effects and doses (e.g. the effects of our lowest concentration of 0.5 μM of ZEA as well as of α -ZOL), although, the quantitative interaction receptor–effective substance (Schmieder et al. 2003) should not be underestimated either. The availability of substances itself could not explain the transitions between the inhibitory and stimulatory effect – e.g. in the cited study of Kim et al. (2014) where they observed a higher motile sperm ratio compared to the control group after 6 hours of incubation. Such effects can be related to the so-called “non-monotonic dose-response curves” defined as a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined (Vandenberg et al. 2012).

CONCLUSION

Our data indicate that the effects of ZEA, α -ZOL, or GEN are already demonstrable with the lowest tested concentrations, i.e. 0.5 μM of the compound in a dose-dependent manner, which shifts the effective concentrations of ZEA, α -ZOL, and GEN to values reachable in the field conditions and raises the question of risk assessment of these compounds in pig reproduction.

Repeated findings of shifted proportions of sperm subpopulations without any significant changes in the mean values of kinematic parameters suggest that under milder stress these mean CASA values need not necessarily be sensitive enough as an indicator of changes in the sperm motility. This should be considered especially in toxicological research on a sperm model where even mild changes can be indicative.

The discussed background to the inconsistency of so far published papers should be taken into account in future similarly focused studies.

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Received: 2017–04–01

Accepted after corrections: 2017–07–24