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Effects of short-term moderate ZEN consumption on uterosacral ligament elasticity in pubertal gilts

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ABSTRACT

Zearalenone (ZEN) is a potent estrogenic toxin in swine, contributing to economic losses in herds via reproductive consequences such as pelvic organ prolapse (POP). To better understand the relationship between ZEN-consumption and reproductive symptoms, an animal feeding study with pubertal gilts was designed. The gilts were exposed to three different treatments: solvent-only feed for 21 days (n = 10), ZEN-spiked feed for 7 days followed by solvent-only feed for 14 days (n = 10), and ZEN-spiked feed for 21 days (n = 10). The gilts did not display any ZEN-related symptoms throughout any of the treatments. At the end of the trial the elastic properties of the USLs from participating gilts were evaluated along two loading directions: main direction (MD) and perpendicular direction (PD). The elastic properties included average stresses at 2% and 4% strains, and secant moduli. Overall the elastic properties of the USLs did not vary across treatment groups or between loading directions. In the MD, average stress increased from 32.96 ± 4.43 kPa at 2% strain to 63.21 ± 9.69 kPa at 4% strain, with a secant modulus of 1.52 ± 0.27 MPa. In the PD, average stress increased from 40.82 ± 4.22 kPa at 2% strain to 83.38 ± 9.17 kPa at 4% strain, with a secant modulus of 2.13 ± 0.31 MPa. Continued research into the relationship between ZEN consumption and reproductive symptoms such as POP is necessary in order to mitigate their deleterious effects in herds.

1. Introduction

Zearalenone (ZEN) is a potent, estrogenic, mycotoxin produced by fungi in the genus *Fusarium*. These fungi contaminate crops such as corn (Munkvold 2003; Taylor et al. 2000), wheat (Schneweis et al. 2002), and soybean (Wang et al. 2010), which are common ingredients in swine feed. ZEN concentrations vary with crop and geographic region, with a typical range of 0.1–1 mg kg^{-1} (Binder et al. 2007). In addition to field crops, ethanol co-products known as dried distiller's grains with solubles (DDGS) are an increasingly common ingredient in swine feed (Agyekum et al. 2014; Jung et al. 2013; Spiehs et al. 2002). Unfortunately, DDGS have been found to contain increased levels of ZEN, at approximately 2.12 mg kg^{-1} (Khatibi et al. 2014). Though no advisory limits currently exist for ZEN in the United States, the concentrations of ZEN reported in DDGS often exceed the 0.1–0.25 mg kg^{-1} limit in swine feed that was set by the European Union in 2016 (Romer Labs 2016). The swine industry has been notably affected by the increased mycotoxin concentrations in DDGS, with a single mycotoxin (fumonisin) in DDGS contributing to financial losses exceeding $147 million annually (Wu and Munkvold 2008).

Swine are notably sensitive to the estrogenic effects of ZEN and may exhibit a wide range of symptoms (Kanora and Maes 2009). These symptoms vary with age and parity of the animal, and may include vulva swelling, anestrous, decreased litter size and weight, and pelvic organ prolapse (POP) (Bryden 2012; Edwards et al. 1987; Kanora and Maes 2009; Minervini and Aquila 2008). While females tend to display symptoms more overtly, males are not immune to ZEN hormonal effects (Zheng et al. 2016), and may experience symptoms such as decreased

Abbreviations: CMOS, complementary metal oxide semiconductor; DDGS, dried distiller's grains with solubles; DIC, digital image correlation; MD, main loading direction; PBS, phosphate buffer solution; PD, perpendicular loading direction; POP, pelvic organ prolapse; USL, uterosacral ligament; ZEN, zearalenone; ER, estrogen receptor

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libido and infertility. Symptoms are often reversed with a change in feed, though frequent changes of feed generate excessive waste and pose financial burden on swine producers.

The relationship between ZEN and the development of POP is of particular concern because of increasing rates of POP in swine herds (Iida et al. 2019; Stock et al. 2017). Afflicted pigs are culled from herds (Benjamin and Yik 2019), contributing financial losses through veterinary care and the lost of future litters. One study estimates losses of $5000–7500 per year, per 1000 sows (Stock et al. 2017). POP, which consists in the displacement of organs from their normal anatomical positions, has been cited as one of the more extreme symptoms of ZEN toxicosis. In a study by Christensen et al., at high ZEN concentrations (500–600 mg kg^{-1}), 3 of 15 participating females developed POP between 25 and 47 days (Christensen et al. 1972). At more moderate ZEN concentrations, similar to those found in DDGS (2 mg kg^{-1}), 7 of 40 participating gilts were found to develop POP within 15 days (Rainey et al. 1990). Similarly, Zimmerman et al. indicated that POP is a symptom of 3–10 mg kg^{-1} ZEN exposure (Zimmerman et al. 2019). Both vaginal and rectal prolapses have been reported as a result of ZEN consumption (Blaney et al. 1984). Research into the mechanical behavior of pelvic floor tissues has helped make significant progress in understanding and treating POP in humans (Abramowitch et al. 2009), and may serve similar benefits to swine production. Further insight into the development and onset of POP in swine may help veterinarians and swine producers to improve reproductive health of the herd, and mitigate the associated financial losses.

ZEN's estrogenic properties may cause structural changes in tissues, contributing to the development of POP. Estrogen is known to affect structural components of tissue, such as collagen, which in turn affect tissue mechanics and function. This phenomenon has been widely explored in the context of human musculoskeletal injuries as well as POP. Notably, in the context musculoskeletal injury, estrogen has been found to affect collagen production and cross-linking, which in turn lead to changes in tissue stiffness (Chidi-Ogbolu and Baar 2019). In addition, research regarding the cardinal ligament from human patients with and without prolapse has indicated a decrease in fibroblast proliferation related to the binding of exogenous 17β-Estradiol to estrogen receptor α (ERα) (Liu et al. 2006). ERα expression has also been found to significantly increase in human patients exhibiting POP (Ewies et al., 2004). ZEN is a full agonist of ERα and a partial agonist of estrogen receptor beta (ERβ) (Kuiper et al. 1998), and may disrupt these endocrine pathways through receptor-binding or interfering with compounds further down the endocrine pathway (Li et al. 2012). Disruption of estrogenic pathways via interaction with ERα or downstream estrogen-pathway activity may therefore cause changes in tissue composition which relate to the POP observed in swine following ZEN consumption.

ZEN consumption also induces growth of reproductive organs. The swine uterus (Gajecka et al., 2012), vagina (Döll et al., 2004), and ovaries (Obremski et al., 2003) have been demonstrated to increase in response to ZEN consumption. Gajecka et al. found increased evidence of cell proliferation in the granulosa cells of the ovary, and they suggested this may be related to ERβ binding activity in this cell type, as ERβ is the most prominent receptor in this tissue. A similar result was reported by Oliver et al. (Oliver et al. 2012), who found that growth of the uterus was associated with increased ERβ expression, despite ERα being the most prominent in this tissue type. The role of ERβ in cell proliferation is not yet clear. The increase in tissue weight as a result of cell proliferation may have consequences for the supportive ligaments in the pelvic floor.

As vaginal and rectal prolapse develop or the weight of the uterus increases due to ZEN consumption, the uterosacral ligaments (USLs), which are the primary pelvic supportive structures of the vagina, cervix, and uterus, may be altered or overstretched. These ligaments are located between the vagina and the rectum, connecting the proximal vagina to the sacrum (Tan et al. 2015). Moreover, estrogen receptors in the USL (Mokrzycki et al. 1997), may be responsible for changes in tissue elasticity (Shahryarinejad et al. 2010). Therefore, mechanical testing that quantifies the amount of stretch that the USLs can sustain under load may serve to determine possible ZEN-induced alterations of these ligaments in swine herds.

To better understand the role of ZEN in the development of swine reproductive disorders, we conducted a feeding trial in which pubertal gilts underwent one of three possible feeding treatments: solvent-only feed for 21 days, ZEN-spiked feed for seven days followed by solvent-only feed for 14 days, or ZEN-spiked feed for 21 days. ZEN-spiked feed contained a ZEN concentration similar to that found in DDGS. Pubertal gilts were selected to exclude factors such as parity and age which may also alter pelvic organs and tissues, and contribute to the development of POP (Reay Jones et al. 2003). At the end of the trial, the elasticity of the USLs was evaluated using planar biaxial testing in combination with digital image correlation (DIC) methods and elasticity measurements were compared across treatment groups. The specific objective of this study was to determine how short-term, moderately dosed ZEN consumption may influence the elasticity of the USLs.

2. Materials and methods

2.1. Animal feeding trial

Methods and procedures for this feeding trial were approved by the Virginia Tech Institutional Care and Use Committee (IACUC). Thirty cross-bred pubertal gilts, approximately six months old, averaging (± standard deviation) 106.9 ± 17.4 kg were housed individually and randomly assigned to one of three treatments, with 10 gilts in each treatment group: (treatment 1) solvent-only feed for 21 days, (treatment 2) ZEN-spiked feed for 7 days followed by solvent-only feed for 14 days, or (treatment 3) ZEN-spiked feed for 21 days (Fig. 1). Each gilt participated in the trial for a total of 21 days, representing the length of a full estrous cycle. This length was selected to allow gilts to begin and end the feeding trial at the same stage of their estrous cycle.

Small, concentrated, portions of ZEN-spiked and solvent-only feed were prepared prior to the feeding study by modifying a commercially available grower diet (Big Spring Mill, Elliston, VA). To make ZEN-spiked feed, 600 mL of 10 mg mL^{-1} ZEN working solution was added to 20 g grower diet. The 10 mg mL^{-1} ZEN solution was prepared by dissolving crystalline ZEN (≥98% purity, J&K Scientific, Beijing, China) in acetonitrile (Thermo-Fisher, Waltham, MA, USA). To make solvent-only feed, 600 mL acetonitrile was added to 20 g of commercial grower diet. Dissolving ZEN in acetonitrile allowed for more efficient control over the amount of ZEN added to feed, while minimizing the safety risks associated with weighing out crystalline ZEN. Analytical methods have demonstrated that ZEN does not readily evaporate alongside acetonitrile (Kinani et al. 2008), and careful measures were taken during pipetting to minimize the chance of ZEN adhering to the container. For both feed types, all added solvent was allowed to evaporate under a fume hood overnight, then the feed was capped, and stored at room temperature in a dark and dry area until feeding. Preliminary studies were conducted to verify that the 6 mg of added ZEN was successfully transferred from the concentrated 20 g portions. ZEN concentrations were determined in the ZEN-spiked and solvent-only concentrated portions of feed using previously validated GC–MS methods (Khatibi et al. 2014). We recovered an average of 5.66 mg ZEN in ZEN-spiked feed, indicating an acceptable average transfer rate of 94%. ZEN concentrations in the solvent-only feed were below the limits of detection, suggesting < 0.1 μg ZEN in the concentrated portion of solvent-only feed.

Immediately prior to feeding each morning, the 20 g concentrated portion of treated feed was combined with 207 g non-treated commercial grower diet to make a 227 g (0.5 lb) “treatment ration” which was fed to the gilts according their treatment group and timeline (Fig. 1). After each gilt had finished consuming the 227 g treatment

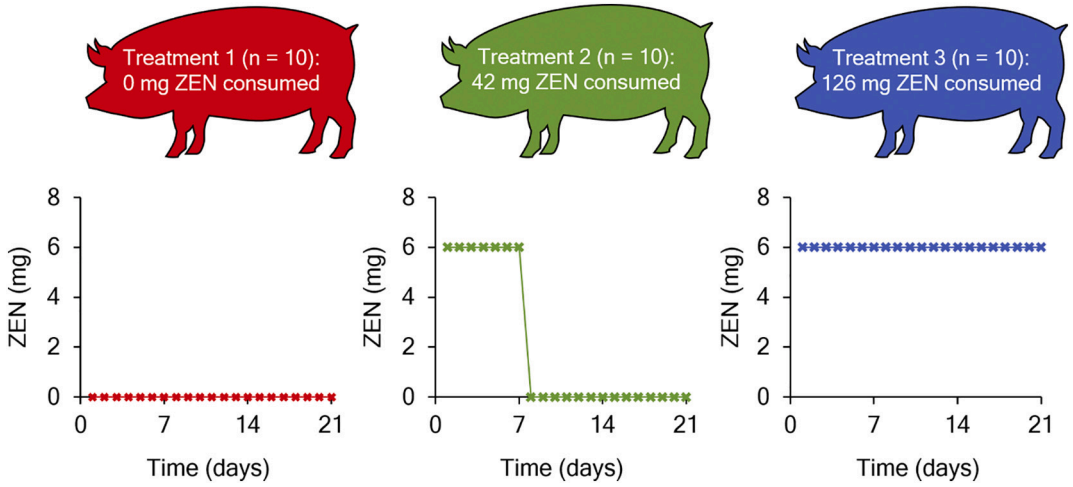

Fig. 1. Total ZEN consumption from gilts in each treatment group is represented in pig shilhouettes above a timeline of ZEN consumption throughout the feeding trial. Thirty gilts were randomly assigned to one of three treatment groups, with 10 gilts in each group. Gilts from treatment 1 group received solvent-only feed for 21 days, gilts from treatment 2 group received ZEN-spiked feed for 7 days, followed by solvent-only feed for 14 days, and gilts from treatment 3 group received ZEN-spiked feed for 21 days. Over the course of the 21-day trial, gilts from treatment 1 group consumed only low-level naturally occurring ZEN from feed, gilts from treatment 2 group consumed 42 mg ZEN, and gilts from treatment 3 group consumed 126 mg ZEN.

ration, the animal was fed an additional 2.04 kg (4.5 lb) of commercial grower diet. Gilts who were given ZEN-spiked feed consumed 6 mg ZEN alongside 2.27 kg (5 lb) feed, for an overall concentration of 2.67 mg kg^{-1} ZEN in feed. This is similar to concentrations of ZEN that have been found in commercially available DDGS (Khatibi et al. 2014). Gilts who were given solvent-only feed may have consumed relatively small amounts of naturally occurring ZEN (< 0.1 μg ZEN). Drinking water was provided to each gilt for *ad libitum* consumption. Throughout the feeding trial, gilts were monitored for symptoms associated with ZEN consumption, such as feed refusal, vulva swelling, and prolapse.

2.2. Specimen collection and preparation

At the end of the trial, gilts were slaughtered at a local abattoir, where tissue was collected. Reproductive tracts were removed from each gilt, along with a portion of the rectum bound to the USL. The mid portion of the USLs that is located between the rectum and proximal vagina was isolated (Fig. 2). A safety pin was used to mark the main loading direction (MD) of the USLs, which is the direction of gravity between the rectum and proximal vagina junction. USL specimens were removed from the rest of the tract and stored in a freezer bag with a few drops of phosphate buffer solution (PBS). They were then frozen at -20 °C until mechanical testing (Baah-Dwomoh et al. 2018).

After the USLs were collected, excess tissue was removed from the reproductive tract in order to isolate the vagina, cervix, uterus, oviducts, and ovaries. The reproductive tract was then weighed.

On the day of testing, the specimen was allowed to gently thaw to room temperature. Rubod et al. demonstrated that freezing and gently thawing specimens of reproductive tissue do not significantly affect tissue mechanics (Rubod et al. 2007). Once thawed, the specimen was submerged for 10 min in a bath of approximately 95% (*v*/v) PBS and 5% (v/v) methylene blue dye (Fisher Science Education, Nazareth, PA). The dyed specimen was laid flat and trimmed to a 30 × 30 mm square, with edges oriented in the MD and the loading direction perpendicular to the MD (PD). When possible, multiple 30 × 30 mm specimens were collected from one gilt. Thickness measurements were taken in four locations along each specimen using a low force digital caliper under 50 g compressive load (Absolute Low Force Caliper Series 573, Mitutoyo, Kawaski, Japan). Specimens were mounted to the biaxial tensile testing apparatus using a tethering technique that allows for an even distribution of force along specimen edges (Macrae et al. 2016). Two pairs of safety pins connected by 4.0 cm fishing line were fastened

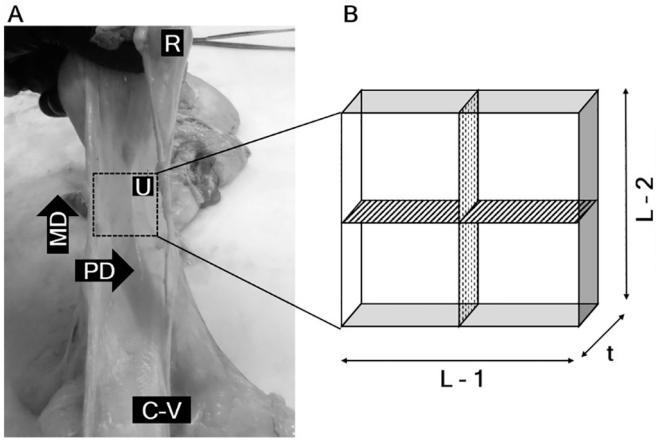

Fig. 2. (A) A front-facing image of the USLs still bound to the rectum (R) and cervix-vagina junction (C–V). The USLs were removed and trimmed to a square 30 × 30 mm specimen (dashed line) with sides oriented in the main direction (MD) and perpendicular direction (PD). (B) A depiction of specimen measurements necessary to calculate the stress in the MD and CD, including: length across the MD (L1) and length across the PD (L2), average specimen thickness (t), and resulting cross-sectional area perpendicular to the MD (striped) and PD (dotted).

equidistantly along each edge of the specimen, for a total of 4 pins along each edge. A speckle pattern suitable for DIC was applied to the surface of the specimen, by spraying white spray paint (Rustoleum, Vernon Hills, IL) through a mesh, over the specimen (Lionello et al. 2014).

2.3. Mechanical testing

The speckled specimen was then mounted to a planar biaxial tensile testing system (Instron, Norwood, MA) by wrapping the fishing line around a set of custom grips, and gently submerging the specimen in a bath of 1× PBS. The system was equipped with 20 N load cells (accuracy ± 0.02 N, Instron, UK). The protocol consisted of three distinct phases: preconditioning, rest, and ramp loading (Fig. 3). During preconditioning, the specimen was cyclically loaded ten times from 0.05 N to 0.5 N at a rate of 0.1 mm s^{-1}. After preconditioning, the specimen was allowed to rest for 10 min. During the ramp loading

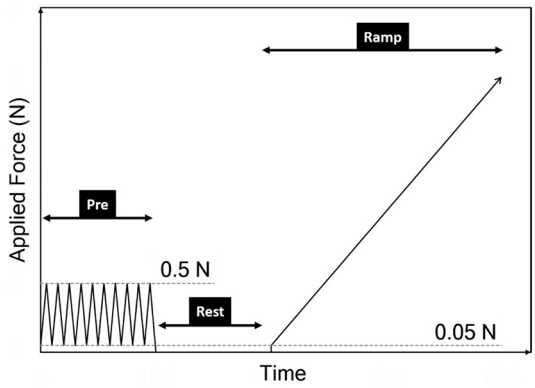

Fig. 3. The three phases of mechanical testing: preconditioning (Pre), rest, and ramp. During preconditioning, the specimen was cyclically loaded from 0.05 N to 0.5 N at 0.1 mm s^{-1}. During rest, the specimen was allowed to rest, with no applied load, for 10 min. During ramp loading, the specimen was loaded starting from 0.05 N then displaced at 0.1 mm s^{-1} until tearing was observed.

phase, the specimen was pre-loaded to 0.05 N, then stretched at 0.1 mm s^{-1} until tearing at the pins was observed.

Force data were recorded only during the ramp loading phase of the testing protocol. Nominal axial stress data in the MD and PD were computed by dividing the corresponding axial force data by the undeformed cross-sectional area that was perpendicular to the MD and PD, respectively. Undeformed cross-sectional area was calculated by multiplying the side length of the specimen by its average thickness (Fig. 2). Side length was determined from specimen images by measuring initial, average distance between opposite pins, using ImageJ (National Institutes of Health, USA). Nominal axial stress will hereafter be referred to as "stress" in either direction.

2.4. Digital imaging correlation

Non-contact strain measurements were performed throughout the ramp loading phase of mechanical testing using a 3D DIC system (Vic-3D, version 8, Correlated Solutions, Columbia, SC). The DIC system consisted of two CMOS cameras (Basler ace acA2440-75 µm, Basler Inc., Exton, PA) fit with c-mount lenses (Xenoplan 2.8/50 Schneider Optics Inc., Hauppauge, NY). High resolution (2448 × 2048 pixels) images were captured at a rate of ten frames per second. Local axial Lagrangian strains in the MD and PD were then calculated over a square region in the center of each specimen, away from the pins, using the DIC system software (Vic-3D, version 8, Correlated Solutions, Columbia, SC) and averaged to compute a single average axial Lagrangian strain value along the MD and a single average Lagrangian strain value along the PD at each time point during testing (Fig. 4). Average axial Lagrangian strain will hereafter be referred to as "strain."

2.5. Statistics and data analysis

Stress-strain curves were generated for each specimen by pairing stress and strain data at similar time points throughout the ramp phase of the protocol. Statistics were performed using Minitab software (version 19.1.1, State College, PA, USA). Average MD and PD stresses were compared at 2% and 4% strains, within each treatment group, and across treatment groups, using repeated measures ANOVA. A line was fit between individual stresses at 2% and 4% strains using Matlab (The MathWorks, Inc., version R2017a, Natick, MA), with the slope of this line representing the secant modulus of each specimen. Secant modulus represents material stiffness, with higher values indicating a stiffer material. The average secant moduli in the MD and PD were compared within each treatment group and across treatment groups, using repeated measures ANOVA. Average stress-strain curves in each direction

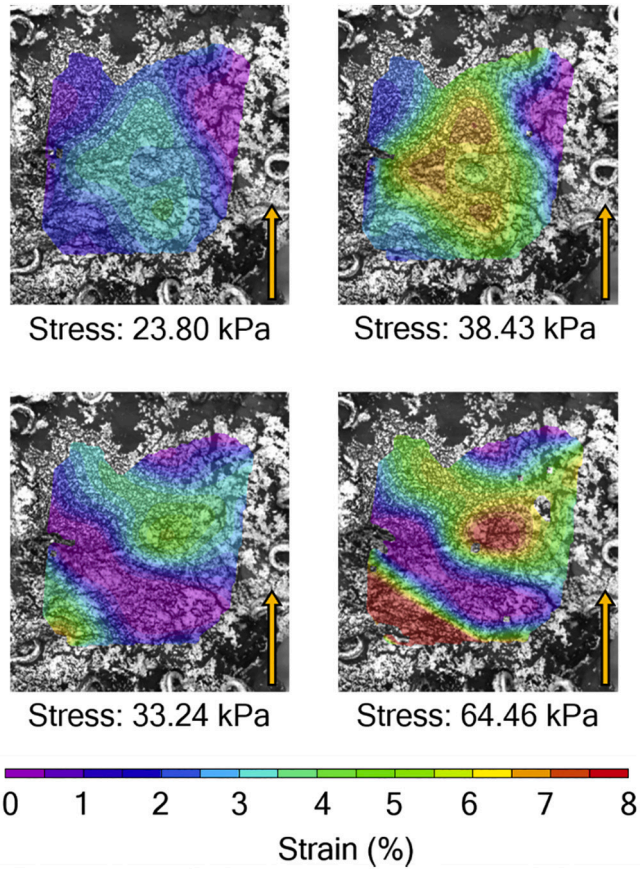

Fig. 4. Strain maps of a representative specimen at 2% (left) and 4% (right) average strains in the MD (top) and PD (bottom) directions with the measured stresses. The yellow arrow indicates the MD, and the color gradient represents strain (%) values ranging from 0% to 8%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for each treatment group and for all treatment groups were generated by comparing average stresses across 0.5% strain intervals from 0 to 4% strain. Reproductive tracts weights were compared using a one-way ANOVA.

3. Results

3.1. Specimen collection and suitability

Of the 30 gilts who participated in the feeding trial, 27 completed it; nine gilts were from treatment 1 group, eight gilts were from the treatment 2 group, and ten gilts were from the treatment 3 group. The three gilts who did not complete the trial were removed for clinical reasons that were not related to ZEN, but interfered with treatment consumption. No reproductive anomalies were observed in gilts from any treatment group.

A total of 27 USLs was collected, generating thirty-two 30 × 30 mm specimens suitable for testing. Images of the specimens were analyzed after mechanical testing to determine if the speckle patterns created for DIC strain measurements were suitably tracked throughout the test. The accuracy of DIC tracking is dependent on a number of factors which could not be evaluated until after testing was complete, including speckle size, distribution, and adherence (Dong and Pan 2017; Palanca et al. 2016). Speckle patterns were successfully tracked on a total of 19 specimens: six specimens from the treatment 1 group, six specimens from the treatment 2 group, and seven specimens from the treatment 3 group (Figs. 5-7). Successful speckle patterns were observed to

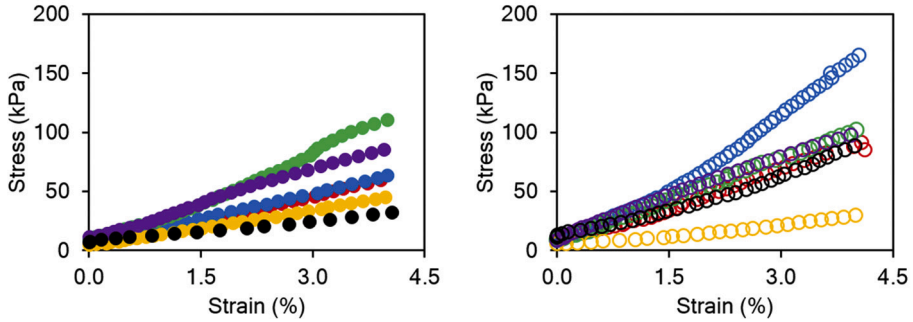

Fig. 5. Individual stress-strain curves from gilts in treatment 1 group in the MD (left, solid circles) and PD (right, hollow circles). Data from the same specimen are represented using the same color in the MD and PD.

deteriorate at high strains, so all strain data were compared at lower strains, up to 4%. DIC strain maps of a representative, successfully speckled specimen, at 2% and 4% strains, in the MD and PD, are shown in Fig. 4. Average stress-strain data are reported in Fig. 8. The average thickness (± standard deviation) of all reported specimens was found to be 0.36 ± 0.12 mm. The cross-sectional area (± standard deviation) in the MD and PD of all reported specimens were found to be 12.08 ± 4.57 mm^2 and 12.12 ± 4.53 mm^2, respectively.

3.2. Reproductive tract weight

Reproductive tract weights were determined for all 27 gilts which completed the feeding trial. No significant differences were determined between the average weight of the reproductive tract of gilts from each treatment group ($p = 0.22$). The average weight of the reproductive tract (± standard deviation) was 517.6 g ± 210.8 g for the treatment 1 group, 599.9 g ± 218.0 g for the treatment 2 group, and 647.6 g ± 248.1 g for the treatment 3 group.

3.3. Elastic properties

Table 1 describes the average stress at comparable strain values of 2% and 4% in the MD and PD. At approximately 2% strain, no significant differences were found between average stress in each treatment group within the MD or PD ($p = 0.23$), nor was any significant difference found overall between the MD and PD ($p = 0.25$). Average stress at 2% strain was qualitatively higher in the PD than MD for USL specimens from treatment 1 and 3 groups, while the opposite result was observed in specimens from treatment 2 group (Table 1). Overall, stress at approximately 2% strain was qualitatively found to be higher in the PD than MD, with 13 specimens following this trend, and 6 specimens following the opposite trend.

At approximately 4% strain, no significant differences were found between average stress in each treatment group within the MD or PD ($p = 0.17$), nor was any significant difference found overall between the MD and PD ($p = 0.18$). Average stress at 4% strain was qualitatively higher in the PD than MD for USL specimens from treatment 1 and 3 groups, while the opposite result was observed in specimens from treatment 2 group (Table 1). Overall, stress at approximately 4% strain was qualitatively found to be higher in the PD than LD, with 12 specimens following this trend, and 7 specimens following the opposite trend.

Fig. 9 illustrates average secant modulus in the MD and PD of specimens from each treatment group, and the overall average secant modulus across the three treatment groups. The average secant modulus was not significantly different between treatment groups within the MD or PD ($p = 0.21$), nor was any significant difference found between the MD and PD overall ($p = 0.20$). The average secant modulus was qualitatively higher in the PD than MD for USL specimens from treatment 1 and 3 groups, while the opposite was observed in specimens from treatment 2 group. Overall, the secant modulus was qualitatively found to be higher in the PD than MD, with 12 specimens following this trend, and 7 specimens demonstrating the opposite trend.

4. Discussion

The objective of this study was to evaluate changes in elasticity of the USLs in gilts that are caused by ZEN-consumption. To our knowledge, this is the first study to investigate the relationship between ZEN-consumption and elastic properties of supportive ligaments of reproductive organs. We characterized the elasticity of the USLs along two primary loading directions (MD and PD) across three groups of gilts, receiving solvent-only feed for 21 days, ZEN-spiked feed for 7 days followed by commercial feed for 14 days, and ZEN-spiked feed for 21 days. Specifically, we evaluated stresses at 2% and 4% strains and secant moduli, and found that these elastic quantities did not vary significantly across treatment groups or loading directions.

The concentrations of ZEN utilized in this trial did not induce changes in elasticity of the USLs (or clinical symptoms). Zimmerman et al. reported that, at concentrations of ZEN comparable to those used

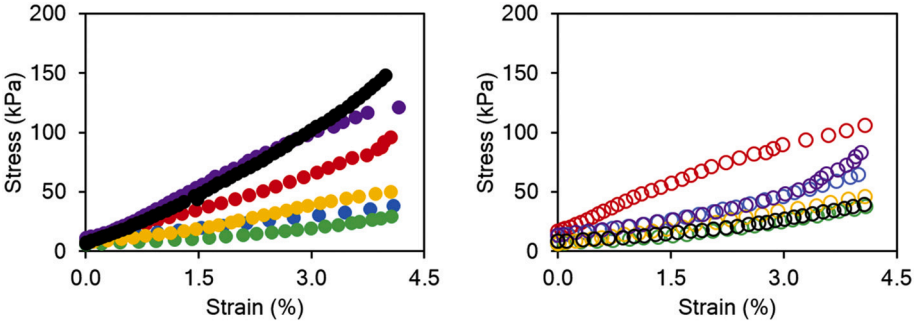

Fig. 6. Individual stress-strain curves from gilts in treatment 2 group in the MD (left, solid circles) and PD (right, hollow circles). Data from the same specimen are represented using the same color in the MD and PD.

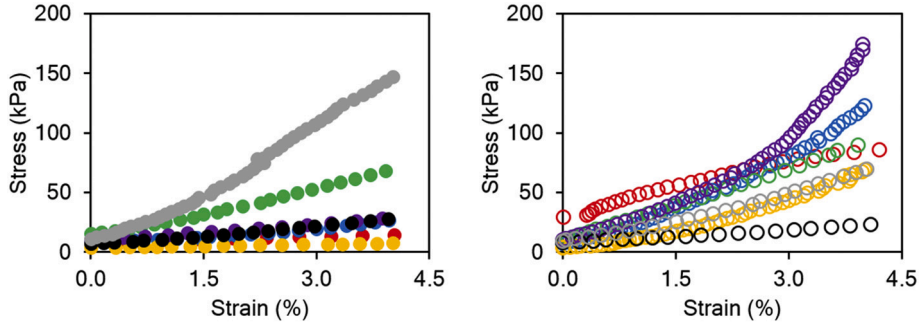

Fig. 7. Individual stress-strain curves from gilts in treatment 3 group in the MD (left, solid circles) and PD (right, hollow circles). Data from the same specimen are represented using the same color in the MD and PD.

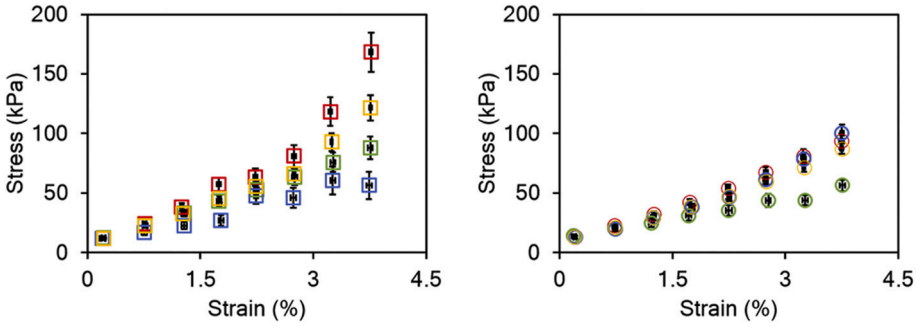

Fig. 8. Average stress-strain curves of USLs from each of the three treatment groups and for all the treatment groups in the MD (left, squares) and PD (right, circles). Data from the treatment 1 group (n = 6) are represented in red, from the treatment 2 group (n = 6) in green, from the treatment 3 group (n = 7) in blue, and from all three treatment groups (n = 19) in yellow. Horizontal and vertical error bars represent S.E.M. strain and stress data, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in this study (3–10 mg kg^{-1}), pubertal gilts experienced POP (Zimmerman et al. 2019). Similarly, Rainey et al. showed that concentrations as low as 2 mg kg^{-1} were sufficient to induce POP. Some studies have reported that, at significantly higher ZEN concentrations in feed (500–600 mg kg^{-1}), POP occurred after 47 days of continued ZEN-consumption (Christensen et al. 1972). In combination with the moderate concentration of ZEN used in our study, the relatively small sample size may also have affected the outcome this trial. POP is a severe symptom of ZEN consumption, but it occurs at relatively low rates. Overall POP rates in sows have been reported to range between 1 and 3% (Stock et al. 2017), while, in a more extreme case related to ZEN *Fusarium* contamination in feed, 40% of a herd was found to display symptoms of POP (Bristol and Djurickovic 1971). The age group and concentration of ZEN used in this study were chosen to reflect realistic concentrations of ZEN which appear in feed (Khatibi et al. 2014) and potentially contribute to the development of POP in a specific age group of swine (Zimmerman et al. 2019). However, the young age and nulliparity of our animals may have influenced our outcomes, as POP is a condition which is currently rising among sows. The lack of significant differences among the elastic properties of the USLs from the three treatment groups is likely due the lack of clinical symptoms in the gilts. Mechanical changes of the USLs simply may not occur until the reproductive system is severely compromised.

ZEN may influence USL mechanics through disruption of estrogenic pathways. It is well understood that hormonal events such as pregnancy and menopause can then alter the resilience of the USLs (Reay Jones et al. 2003). ZEN-consumption mimics these hormonal events through interactions with ERα or ERβ pathways, which can alter reproductive tissue composition and weight (Kowalska et al. 2016; Kuiper et al. 1998). Previous studies have reported an increase in weight of the reproductive tract following ZEN consumption (Jiang et al. 2010; Oliver et al. 2012; Teixeira et al. 2011). This increased weight may be attributed to increased cell proliferation, as has been observed in the ovaries and uterus (Gajecka et al. 2012, Gajecka et al., 2011). The increased reproductive tract weight reported in other studies may affect the stability of supportive tissues such as the USLs. Alternatively, the interaction of ZEN with ERα may affect collagen content and stability within the USL. ERα has been found to affect fibroblast proliferation in the cardinal ligament, a neighboring pelvic support tissue (Liu et al. 2006). Changes in collagen content have been well documented in relation to human POP (Gong and Xia 2019). In this study, no differences were observed between gilts who did and did not consume ZEN, regarding reproductive tissue weight and USL elasticity. However, further research into the mechanical relationship between ZEN and POP may lead to more information on how endocrine disruption affects function of pelvic supportive tissues such as the USL.

Table 1
Average strain and stress in the MD and PD for each treatment group, and overall (± S.E.M.)

		2% strain		4% strain		
Treatment group	Direction	Average strain (%)	Average stress (kPa)	Average strain (%)	Average stress (kPa)	Secant modulus (MPa)
1: no added ZEN (n = 6)	MD	2.06 ± 0.01	35.88 ± 5.26	3.98 ± 0.02	66.26 ± 10.53	1.58 ± 0.28
	PD	2.03 ± 0.01	48.14 ± 7.13	4.01 ± 0.02	95.68 ± 16.08	2.39 ± 0.48
2: ZEN for 7 days (n = 6)	MD	2.03 ± 0.02	40.49 ± 8.64	4.06 ± 0.02	80.55 ± 18.04	1.96 ± 0.49
	PD	2.04 ± 0.02	32.60 ± 7.44	4.06 ± 0.01	62.66 ± 10.19	1.48 ± 0.21
3: ZEN for 21 days (n = 7)	MD	2.02 ± 0.05	24.01 ± 7.07	3.97 ± 0.02	45.72 ± 17.03	1.10 ± 0.51
	PD	2.01 ± 0.01	41.59 ± 6.20	4.04 ± 0.03	90.58 ± 16.57	2.45 ± 0.64
Overall	MD	2.04 ± 0.02	32.96 ± 4.43	4.00 ± 0.02	63.21 ± 9.69	1.52 ± 0.27
	PD	2.03 ± 0.01	40.82 ± 4.22	4.04 ± 0.01	83.38 ± 9.17	2.13 ± 0.31

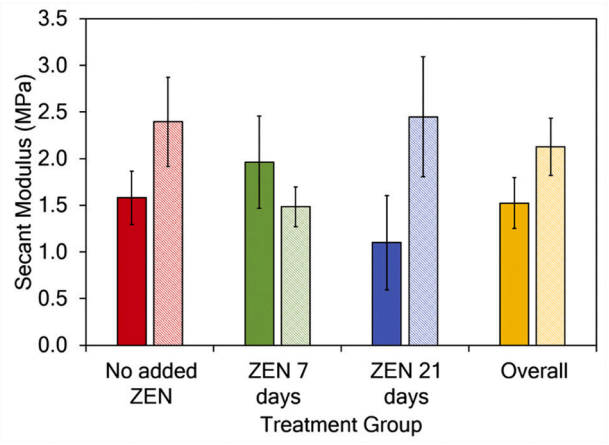

Fig. 9. A bar plot showing average secant moduli of the USLs from gilts in each of the three treatment groups, and the overall average secant modulus across all groups. Data from the treatment 1 group (n = 6) are represented in red, from the treatment 2 group (n = 6) in green, from the treatment 3 group (n = 7) in blue, and from all treatment groups in yellow (n = 19). Solid bars represent the MD and cross-hatched bars represent the PD. Error bars represent S.E.M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Mechanical characterization of pelvic tissues is commonly employed for studying the deleterious effects of POP in humans [48]. Swine are a popular large animal model for studying the USLs due to established similarities in histological and mechanical properties between swine and human USLs (Baah-Dwomoh et al. 2018; Becker and De Vita 2015; Tan et al. 2016, 2015). In our study, we found that the elastic properties of USLs from gilts, at small strains (2% and 4%), are not significantly different between the MD and PD. Recent research conducted on USLs from sows supports this finding, showing similar elastic properties in the MD and PD of USLs (strains < 3.5%) (Baah-Dwomoh et al. 2018). The values of the secant moduli and overall stresses experienced at 2% and 4% strains were also comparable to those previously reported for sows (Baah-Dwomoh et al. 2018). It is possible that, at higher strains (> 4%), the elastic properties of the USLs may differ in the MD and PD. As the strain and stress increase during mechanical testing, more collagen fibers within the USLs are recruited and straighten. Changes in fiber architecture may then lead to significant differences in the elastic properties. Additional work is needed to establish potential differences in the mechanical properties of the USLs in the MD and PD.

Uniaxial and biaxial tensile techniques are commonly employed when investigating USL mechanics in human (Baah-Dwomoh et al. 2016). The USLs provides multi-directional support to reproductive organs, so biaxial tensile testing is preferred over uniaxial tensile testing because it better reflects the *in vivo* physiological loading conditions of the ligaments. We used the DIC method to compute strain across the surface of the specimen, which helps in computing strain for an inhomogeneous tissue. Fig. 4 demonstrates that strain is not homogeneous across the whole specimen, perhaps due to the pins used for clamping (Eilaghi et al. 2009). For this reason, a region near the center of each specimen, away from pins, was chosen to measure local strains and average values of such strains across the entire region were reported in our results. The DIC technique, however, does have some limitations in that it requires that a random-dot-pattern is maintained throughout the test. In a hydrated environment, the pattern that is created on the surface of the specimen may deteriorate during testing, preventing the accurate measurement of strain. Speckle size, density, arrangement, and adherence can all influence the accuracy of strain measurements (Dong and Pan 2017; Palanca et al. 2016). When investigating the mechanical properties of the USLs at higher strains, alternative speckling methods may be needed.

In this study, we investigated the effects of short-term ZEN consumption at concentrations similar to those found in DDGS. DDGS are known to contain high concentrations of mycotoxins (Khatibi et al. 2014), but the exact reason for this increased mycotoxin concentration is not well understood. Previous work investigating the accumulation of mycotoxins during ethanol distillation alone did not find a substantial increase in ZEN concentration in DDGS (Dzuman et al. 2016), though surveys show very high ZEN concentrations in the DDGS being sold to feed mills (Khatibi et al. 2014). Storage conditions may play a role in these conflicting results (Bryden 2012; Neme and Mohammed 2017), with dark, damp, conditions allowing for proliferation of fungi from the genus *Fusarium*, over time. With increased risk of ZEN contamination through DDGS, incorporation in feed may add risk to the reproductive health of herds.

A variety of factors may have affected the outcome of our study, where gilts did not experience any reproductive anomalies after 7 or 21 days of 2.67 mg kg^{-1} ZEN consumption. These factors include ZEN concentrations, sample size, timeline, age, genetics, and parity, any of which may have influenced the sensitivity of pigs to the increased ZEN concentrations in their diets. Future studies may investigate the concentration of ZEN in swine tissues (Pack et al. 2020), estrogen receptor interactions, and the effects of puberty and parity on swine USL mechanics. Overall, more research is necessary to fully understand the risks associated with feeding ZEN-contaminated diets, and how it may be connected to recent incidents of POP and reproductive anomalies in herds.

5. Conclusions

We have found that concentrations of ZEN, which are comparable to those reported in DDGS, did not cause significant change in the elasticity of the USLs in pubertal gilts after 21 days. The USLs support the vagina, cervix, and uterus, suggesting a role in ZEN-induced forms of POP or reproductive anomalies (e.g., increase in reproductive tract weight). However, no clinical symptoms that affect the reproductive organs were observed in any gilts participating in this trial. Mechanical analysis of the USLs showed that average stresses and secant moduli were not significantly different in two loading directions (MD and PD), and they did not vary significantly between ZEN treatment groups. A variety of factors such as dosage, timeline, age, genetics and parity may have influenced the results of this trial. Further research is warranted to better understand the effects of ZEN on reproductive health, including the rising frequency of POP in swine herds.

Declaration of Competing Interest

None.

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