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Effects of prolonged exposure to zearalenone on the systemic health of dairy cows

Diploma thesis

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Declaration

I hereby declare that I elaborated this diploma thesis independently using the cited literature.

Date

5.2.2024

Signature

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Abstract

Zearalenone, a mycotoxin produced by fungi of genus *Fusarium*, is known to be a threat to not only food and feed safety but also to livestock's health. There is not much knowledge about effects of prolonged exposure to zearalenone in low dosages on dairy cows health. This thesis gives a review about zearalenone and its effects on the body and reports our research on whether zearalenone in low dosage has any effects on selected systemic parameters of dairy cows during long term exposure. In the experiment, 6 dairy cows were fed with dosages of zearalenone below the EFSA (European Food Safety Authorit) regulatory limits (400 ppb/kg DM) for 21 days. Systemic health parameters were measured, namely body temperature, heart rate, respiratory rate, rumen peristalsis and fecal consistency twice a week on two conecutive days, additionally feeding behavior and reproductive parameters were controlled. Although no effects were found for reproductive health or feeding behavior, nearly all of the clinical health parameters were significantly affected by the treatment. Most of parameters were higher in the afternoon. Values increased until week two and then declined which could be a immunogical reaction to zearalenone treatment with a following habituational effect. Heart rate was the one parameter affected the most. The results show that feed contamination with zearalenone, even at low levels, is relevant for the systemic health of dairy cows.

Abstract

Zearalenon, ein Mykotoxin, das von Pilzen der Gattung *Fusarium* produziert wird, ist bekannt dafür, nicht nur eine Gefahr für Lebensmittel- und Futtermittelsicherheit zu sein, sondern auch die Gesundheit von Nutztieren zu beeinträchtigen. Es ist allerdings noch wenig über die Auswirkung einer Langzeitexposition von Zearalenon in niedriger Dosierung auf den allgemeinen Gesundheitszustand von Milchkühen bekannt. Diese Diplomarbeit gibt einen Überblick über Zeralenon und die Auswirkungen auf den Körper und beinhaltet einen Bericht über unsere Forschungsarbeit zu der Fragestellung, ob Zearalenon in niedriger Dosierung bei Langzeitexposition Auswirkungen auf ausgewählte systemische Parameter hat. In dem Experiment wurden 6 Milchkühe über 21 Tage mit Zearalenon in Dosierungen unterhalb der

EFSA (European Food Safety Authority)-Grenzwerte (400ppb/kg TS) gefüttert. Klinische Parameter wurden zweimal pro Woche zweimal am Tag gemessen, nämlich Körpertemperatur, Herzfrequenz, Atemfrequenz, Pansenmotorik und Kotkonsistenz. Zusätzlich wurde das Fressverhalten und der Reproduktionstrakt kontrolliert. Es wurden keine Auswirkungen auf Reproduktionstrakt oder Fressverhalten gefunden, jedoch wurden fast alle klinischen Parameter beeinflusst. Die meisten Parameter waren am Nachmittag höher. Außerdem stiegen die Werte bis zur zweiten Woche an, gefolg von einem Abfall auf das Niveau zu Beginn, was ein Hinweis auf eine immunologische Reaktion mit anschließendem Gewöhnungseffekt sein könnte. Die Herzfrequenz wurde am stärksten beeinflusst. Die Ergebnisse zeigen, dass die Kontamination von Futtermittel mit Zearalenon selbst in kleinen Mengen eine hohe Relevanz hat.

Abbreviations

BW ..... *body weight*
DF ..... *degrees of freedom*
DM ..... *dry matter*
EFSA ..... *European Food Safety Authority*
F. ..... *Fusarium*
FRL ..... *free rumen liquid*
LD50 ..... *average lethal dose*
PARL ..... *particle-associated rumen liquid*
ROS ..... *reactive oxygen species*
SCFA ..... *short-chained fatty acids*
SE ..... *standard error*
TMR ..... *total mixed ration*
ZEN ..... *Zearalenone*
ZOL ..... *α-zearalenol*
α-ZOL ..... *α-zearalenol*
β-ZOL ..... *β-zearalenol*

Table of contents

1 Introduction

Molds and their mycotoxins as contaminants of feed pose health risks to animals and also humans consuming animal products (1). Zearalenone (ZEN), a mycotoxin produced by fungi of genus *Fusarium* (*F.*), is known for its effects on reproductive tract as a mycoestrogen, being able to bind to estrogen receptors (2). Due to microbial degradation and their detoxifying potential, ruminants are almost resistant to mycotoxins in general (3). In case of ZEN, this mycotoxin cannot be totally degraded in the rumen (4). Furthermore, in the rumen most of it is converted to a more active metabolite, α-zearalenol (α-ZOL) (5).

Still, ruminants can excrete also the less potent product, β-zearalenol (β-ZOL) due to further degradation in liver and intestine (6). This means that, in case of dairy cows, the effects of ZEN on their health and the transmission to products for human consumption are estimated as a rather low hazard although at first a more potent derivative is formed in the rumen (7). In this context it is a precondition that the metabolism of the individual is properly working, which means a good overall health condition should be maintained through optimal feeding and husbandry. Furthermore, since effects are also dependent on the dose, the threshold value for ZEN in feeds established by EU (0.5mg ZEN per kg of daily ratio with dry matter of 88%) should not be exceeded to ensure healthy animals and food safety (8).

When cows consume dosages of ZEN higher than the threshold value there can be a harmful impact on animal health. Furthermore, hyperestrogenic effects can occur, like infertility, changes of the reproductive tract (for example alterations of the ovaries and of the endometrium) and even transmission of ZEN to milk (9, 10). Other effects described for different species are hepatotoxic, haematotoxic, immunotoxic and genotoxic consequences (11).

There are already studies about the effects of ZEN on dairy cows health after intake of high dosages over short term periods. In this study, we were especially interested if there are effects on diary cows' clinical health parameters during prolonged exposure to ZEN with concentrations below the EU threshold value, as contamination of feeds with ZEN can often be found in at least small amounts. We focused on heart rate, respiratory rate and body temperature as general health parameters to look for signs of intoxication and inflammation processes.

Furthermore, we concentrated on the gastrointestinal tract by measuring rumen peristalsis, fecal consistency and chewing activity to control the effect of ZEN on the digestive process and the gut functionality. For an additional subset of cows, rectal palpation and ultrasound of uterus and ovaries was additionally performed to see if there were effects on the reproductive tract.

Based on information found in previous works, that will be discussed in detail in the following sections, and due to dosages used below EU threshold level, we would not expect our treatment to have any effects on dairy cows health.

1.1 Mycotoxins

Some fungal species are able to produce mycotoxins, secondary metabolites used to protect themselves and to defend their habitat. Those products can get into the food chain when crops are contaminated by mold either before or after being harvested. Contaminated food or feed represents a highly relevant safety concern as it can cause mycotoxicosis to animals and humans with different effects on health and performance (1).

Over 400 mycotoxins have been discovered already, the most important ones being aflatoxins, ochratoxins and fusarium toxins. The number of species being able to produce toxins is large, however the relevant species that are thought to cause most of food spoilage are considered to be less than ten (1).

Some toxins are more specific than others concerning the genera of fungi they are being produced by. For example, aflatoxin is only produced by genus *Aspergillus*, while fungi of genera *Aspergillus* and *Penicillium* are both able to generate ochratoxins. This also means that some species are able to produce more than one kind of mycotoxin (12).

As previously mentioned, crops can be contaminated with fungi (or molds) while still being on the field (manly *Fusarium*) or while being stored (mainly *Aspergillus* and *Penicillium*). While most mold species are being destroyed during the process of cooking, their mycotoxins can often still cause intoxications (1). The ability of each species to grow and the following contamination with mycotoxins depend on many factors, like temperature or humidity, which highlight again the importance of professional farming practices, processing and storage (12).

The severeness of intoxication is determined by the kind of mycotoxin, duration and amount of exposure, and characteristics of the exposed animal regarding health and fitness, age, sex as well as genetics (2).

1.2 Zearalenone

Besides of deoxynivalenol, one of the most important (and also most frequently detected) mycotoxins of the genus *Fusarium* is zearalenone. Anaylsing the name already gives much information about this metabolite:

- First Gibberella zeae, the teleomorph of *Fusarium graminearum*, (*zea*)
- second resorcylic acid lactone, (*ral*)
- -ene representing the double bond of C-1' to C-2 (*en*)
- and the ending -one standing for C-6' ketone (*one*) (13)

Not only *F. graminearum*, but also *F. culmorum*, *F.equiseti*, *F. cerealis*, *F. semitectum* and *F. crookwellense* are able to synthesize ZEN, with the first two species mentioned being the main producers. All those species of genus *Fusarium* are highly relevant as common contaminants of crops all over the world (11).

However, ZEN is not a typical life-threatening mycotoxin as it does not cause severe illnesses. Still, it is of high relevance as its main effect relies on the similarity to 17β-estradiol, a form of the sex hormone estradiol (2). This fact enables it to bind to estrogen receptors. The consequences are therefore clear: due to the estrogenic effects it can cause hyperestrogenism with all the symptoms of increased estrogen levels, such as swelling of the vulva, ovarial dysfunction or endometrial changes, disrupted conception, abortion and other reproductive problems. For this reason, ZEN is often referred to as mycoestrogen (2). The different impacts on animal and human health and economic problems will be discussed in the sections 1.1.4 and 1.1.5.

1.3 Metabolism, characteristics and mechanism of action of ZEN in mammals

After oral uptake, ZEN is transported to the forestomachs and intestine in ruminants. Contrary to other species in which mycotoxins are rapidly absorbed and metabolized mainly in the liver and also in kidney and intestinal tissue, in ruminants the biotransformation already starts in the rumen (14, 3). Thanks to the rumen microbes which are known to have detoxifying potential, ruminants are relatively resistant to mycotoxins, especially to *Fusarium* toxins, provided that the rumen is well working and with a healthy microbial composition, which highlights again the importance of a good health condition of the animal (3). However, in case of ZEN contamination the microbiome cannot totally detoxify this mycotoxin so that it is further transported to the intestine and biotransformed in the liver (4).

According to Olsen et al. (15) ZEN can be converted in two different pathways:

1. Reduction via hydroxylation, catalyzed by 3α- and 3β-hydroxy-steroid dehydrogenases forming phase I metabolites
2. Conjugation of ZEN and its reduced phase I metabolites with glucuronic acid, catalyzed by uridine diphosphate glucuronyl transferases, forming phase II metabolites

The outcome after reduction are 5 phase I metabolites: α-zearalenol (ZOL), β-ZOL, α-zearalanol, β-zearalanol and zearalanone (16).

These metabolites do not only differ in their structure but also in estrogenic potency through their binding affinity to estrogen receptors. The European Food Safety Authority (EFSA) [16] investigated *in vivo* estrogenic potency by assigning a potency factor relative to ZEN for each phase I metabolite. Results showed that α-ZOL is 60 times more potent than ZEN, while β-ZOL only reaches factor 0.2.

For this reason, α-ZOL and β-ZOL are also referred to as activating and inactivating derivatives (17).

According to Frizzell et al. (18) and other studies, agonist response to estrogen receptor occurs in the order 17β-estradiol > α-ZOL > ZEN > β-ZOL and phase II metabolites as glucuronide conjugates showing the lowest potency. Meanwhile, Blankenship et al. (19) researched on binding of ZEN to bovine endometrial estrogen receptors stating that affinity of ZEN was 2-3 times higher than 17β-estradiol. Since this research was done nearly 40 years ago, it would be

advisable to investigate the ZEN binding dynamics in cattle using more advanced and modern techniques to further validate these findings.

The animal species and the location of conversion in the body will determine which one of the derivates is dominantly formed.

As previously mentioned, in a well working rumen most of ZEN can be degraded to zearalenol by rumen microbes, as it has been proven in several *in vivo* and *in vitro* studies on rumen fluid [3; 4; 5; 6; 7; 14;]. In this part of the gastrointestinal tract, the predominant metabolite produced is α-ZOL (5).

Comparing the conversion of ZEN in livers of five different species, Malekinejad et al. (17) stated that pigs livers microsomes produced the highest amount of α-ZOL, followed by chicken and sheep, while cattle and rats produced the lowest. In case of β-ZOL, the highest amount was found in chicken, the lowest again in cattle. For cattle, β-ZOL seemed to be the dominant hepatic metabolite. A higher amount of β-ZOL was found also in bile (20), highlighting biliary excretion and enterohepatic cycle as important parts of further degradation to less potent forms of ZEN.

Intestinal tissue is one main location for pigs and humans to degrade ZEN (11). Dänicke at al. (3) researched on recovery of ZEN and its metabolites in duodenal digesta in cows: ZEN, α-ZOL and β-ZOL were found, with β-ZOL being of highest concentrations. Meanwhile, ZEN levels in faeces, which counts as a major excretion pathway (21), were relatively steady when ZEN intake was increased with higher concentrations of β-ZOL than ZEN and α-ZOL. This can be ascribed to further degradation by intestinal tissue and liver (6).

Concerning excretion, ZEN and its metabolites can be found not only in faeces but also in urine. Urine of heifers that have been fed with ZEN contaminated feed for 84 days showed that 80% of ZEN was converted to β-ZOL, with the rest being α-ZOL and glucoronidation products. The urinary system has shown to represent 33% of ZEN excretion (22).

As milk is another excretion pathway of the body and at the same time can reach humans for direct consumption, it is important to research on the amount of ZEN and its metabolites in milk. Fink-Gemmels (23) stated that in general, excretion of mycotoxins via milk is rather low, even if the blood-udder barrier is harmed in case of systemic or local infection.

Very little amount of ZEN will be incorporated into the liver (22). In kidneys, bladder, dorsal fat, liver and muscles of male bovine with daily intakes of 0.1 mg ZEN/kg, no ZEN could be found (11).

1.4 Clinical effects of ZEN on health and performance of dairy cows and other species

ZEN is known to have rather limited acute toxic effects. Flannigan (24) investigated the average lethal dose (LD50) in rats and mice after oral uptake with results between 2,000 and 20,000 mg/kg body weight.

Harmful effects rather develop from the interaction with estrogen receptors, implying that ZEN is more relevant for its chronic toxicity. Chronic ZEN intoxication with consequent hyperestrogenism leads to symptoms concerning the reproductive tract and mammary glands. Effects are always depending on dose and time of exposure, on animal species and type of mycotoxin, as well as feeding and husbandry condition, health and reproductive status of the individual.

The most susceptible species is pig, especially young gilts, as many studies have shown. A dose of 1-5 mg/kg of ZEN is enough to show clinical symptoms in gilts, like hyperaemia and edematous swelling of the vulva and sometime even vaginal or rectal prolapse. Furthermore, cycling animals showed pseudopregnancy, nymphomania, atrophy of ovaries and alterations of the endometrium (25). When pregnant, ZEN can lead to embryonic death, decrease of fetal body weight and teratogenic effects on genitals in piglets. In boars, depression of testosterone levels, testes weight and spermatogenesis as well as feminization and suppression of libido are reported (26).

ZEN does not only have effects on reproductive system but also on other organs and systems. ZEN has shown to have haematotoxic effects, as hematological parameters changed when female rats received ZEN intraperitoneally: increase of hematocrit and MCV, decrease of platelet count affecting coagulation of blood, increase of white blood cells. Furthermore, serum specific biochemical parameters were affected: serum creatinine decreased, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and bilirubin increased, which means that ZEN might have an impact on the liver function as well (27).

ZEN also affects the immune system and shows immune toxicity. Mechanisms like intestinal oxidative stress, apoptosis and autophagy are reported (28–30). Marin et al. (31) suggest a possible stimulation of cytokine production and therefore proinflammatory effects in thymoma cell line in vitro. Impact on immunity can occur also via damage of immune organs in mice, like spleen injuries (32). In high concentrations immunosuppression was observed in human peripheral blood mononuclear cells (33).

Additionally, Genotoxic effects on bovine and murine lymphocytes were found (34, 35). Also, carcinogenicity is being discussed. According to the International Agency for Research on Cancer, there is not enough evidence of ZEN inducing cancer (36). However, there is a study about this effect that is worth to mention about ZEN being a possible promotor of mammary tumor in humans due to its stimulation of estrogen receptors (37).

Focusing especially on cattle, systemic health and ruminal ecosystem can be harmed by ZEN. Hartinger et al. (38) investigated effects of ZEN after short-term exposure by feeding six cows 5 mg of ZEN per day. A systemic impact was observed, as body temperature increased to a mild fever which could be explained as an immunological reaction to the mycotoxin. No effects on heart rate or respiratory rate were registered. Also feeding and chewing behaviour were investigated: During ZEN exposure cows showed decreased eating periods per kg dry matter (DM) while chewing index (ruminating + eating time divided by DM intake) and chews per ingested bolus increased as well as the amount of DM intake. There was no effect on total eating and eating time nor on rumination activity. Concerning the rumen, pH and concentration of short-chained fatty acids (SCFA) decreased while the cows showed higher rumination activity. When rumen microbial composition was analysed, *Lachnospiraceae* and *Prevotellaceae* were lower, which are both essential for physiological function of the rumen. This means that ZEN could have effects on microbes through changing the ruminal environment. A further study about short-term effects on rumen microbiota and fermentation processes on Holstein cows (rumen-cannulated) after a single oral bolus of ZEN showed reduction of microbial diversity in free rumen liquid (FRL) but not in particle-associated rumen liquid (PARL), which reinforces the assumption of negative effects of ZEN on microbes, especially bacteria. At the same time, the amount of protozoa increased in PARL, presumably due to their considerable biodegradation capacity (39). Another study showed increased ammonia concentration and at

the same time reduced duodenal flow of microbial protein and utilizable protein after ZEN intake, indicating that microbes were not able to use ammonia (3).

Other economically relevant effects are those on reproductive efficiency and milk yield. Infertility, reduced milk yield and hyperestrogenism are related to ZEN uptake in cows. Weaver et al. (9) fed heifers 250 mg ZEN per day through 3 estrous cycles to investigate their fertility through artificial insemination after 1 cycle and could detect that conception rate did decline. In dairy cows, ZEN concentrations up to 500 mg per day did not have an impact on health, only increased hemoglobin concentrations and smaller size of corpora lutea could be found (40). Also Silva et al. (41) could neither determine changes in the morphometry of the reproductive system nor in plasma estrogen levels at concentrations of 300 ppb ZEN, but the oocyte quality was negatively affected. Similarly, negative impact on the maturation of oocytes was found during ZEN treatment (42). Furthermore, in vitro evaluations showed that at presence of ZEN, sperm-triggered inflammation of bovine endometrial epithelial cells as well as phagocytosis for sperm is increased, while sperm motility is decreased (43). Concerning mammary glands, ZEN can be detrimental by inducing apoptosis via endoplasmic reticulum stress, increasing reactive oxygen species (ROS) and decreasing cell viability and mitochondrial membrane potential (28). This may lead to reduced milk production, although significant studies are missing.

The mentioned studies reinforce the assumption that oocyst quality, conception, fertility and milk production can be negatively affected by ZEN and can pose health risks and economic deficit. However, cattle seems to be rather resistant to ZEN compared to other species.

This seems to be because the metabolite that is mainly formed at the end of all the metabolization and excretion pathways is β-ZOL, which is of less estrogenic potential. Still, ruminal metabolization of ZEN will increase estrogenic potency due to α-ZOL being the dominantly produced and also most potent derivate (11). This means in case of ZEN ruminal degradation does not result in detoxification contrary to other mycotoxins (4). Studies also have proven that with health problems following reduced metabolism or with high oral uptake more ZEN and metabolites will be excreted. For this reason, it is important to follow recommended critical values for ZEN in feeds by EU which is 0.5 mg ZEN per kg of daily ratio with dry matter of 88% for adult cattle in optimal health, feeding and husbandry conditions (8).

However, the studies that investigated the effects of ZEN on the clinical health parameters and on the gastrointestinal functionality of dairy cows were mostly performed in short periods, and the effects of prolonged exposure to ZEN contaminated feed on these parameters warrants further research.

1.5 Relevance of ZEN as a contaminant in dairy cow feed

As mentioned in previous sections, contamination of feedstuffs with ZEN can be found frequently. Studies report about 60-80% of animal feeds containing ZEN concentrations above detection limits and 25% with concentrations above EU threshold value (44). In Austria the situation is comparable to the rest of the world. When complete diet of lactating cows from 100 dairy farms in Austria were analysed for mycotoxins and other contaminants, ZEN was found in 77% of feed samples (45). Another study analysed feed samples from different farms of swine and cattle herds with reports of appropriate case history (for cattle herds: ovarian cysts, decreased slaughter weight, enteritis, feed refuse), showing 67% correlation of case history with positive mycotoxicological results for toxins of fungal species *Fusarium* (46). This means that ZEN contamination of feedstuffs for dairy cows is a current problem in Austria and can also negatively impair heard health.

Concerning the relevance of ZEN contamination for animal production, the main focus is on the negative impact on health of the animals, as well as on economic consequences such as reduced reproductive performance and milk yield. Another highly important effect is the possible transition to animal products which may represent a health hazard to humans (23). However, ZEN degradation processes in ruminants causing the excretion of the less potent metabolite β-ZOL will decrease the risk of effects in the consumer, as previously stated. Usually, high concentrations and experimental conditions are needed to observe effects on human health. Consequently, ZEN is stated as having no importance as a food contaminant under non-experimental conditions, but still having relevant impacts on animal health (7).

Therefore, the aim of this study was to assess the impact of prolonged ZEN exposure on selected health parameters of dairy cows.

2 Material and Methods

2.1 Animals, diet, timeline

The project took place at the University´s dairy farm (Vetfarm) located in Pottenstein in Austria during March and April 2023. The experiment was approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna, in accordance with the University's Guidelines for Good Scientific Practice and authorized by the Austrian Federal Ministry of Education, Science and Research (ref 2023-0.062.024) in accordance with current legislation. This project was part of a larger animal trial involving 18 animals. The 6 cows selected for this experiment were of different ages (3,8±1,8 years) and parity (2,7±1,6 calves), all of them lactating during the experiment (103±30 days in milk at the beginning of the experiment). One of them was breed *Brown Swiss*, the other 5 were *Fleckvieh* (Austrian Simmental). The barn at the University farm is a free-stall barn and during the project the cows were housed in an area with deep litter cubicles and an outdoor zone. The cows had *ad libitum* access to water and to mineral blocks and were fed via special feed bunks (Insentec B.V., Emmeloord, the Netherlands) which opened after contact with the appropriate sensor worn by each of the cows. This provided individual feeding troughs with *ad libitum* access for each cow and automated control and record of each animal´s feed intake.

A total mixed ration (TMR) was provided with 60% forage and 40% concentrate. The exact composition of the diet was 40% corn silage, 20% grass silage, and 40% concentrate (21% Rindastar SM VET (Schaumann GmbH) and 19% Rindastar 39XP (Schaumann GmbH). The ration was mixed and fed twice a day (7:00 a.m. and 2:00 p.m.) with an automatic feeding machine (Trioliet Triomatic T15, Oldenzaal, the Netherlands). Every morning, orts were removed manually from every feed bunk before feeding. Feed intake per cow per day was recorded automatically. In addition, feed consumption was monitored daily by multiple visits to the barn and intake was also recorded manually. TMR was controlled for the amount of natural contamination with ZEN at the beginning and at the end of experiment. Small amounts were found, but the daily intake never exceeded the EFSA recommended threshold, so that any possible effects can be led back to experimental contamination.

The project lasted for 33 days and started with an adaption period of 5 days. The timeline of the experiment is presented in detail in **table 1**. Weights of the cows were taken (body weight (BW) 574±164 kg) at the beginning of the trial, and the animals were allowed to familiarize with the feed bunks during the adaptation. During this period they received TMR only. Chewing activity was measured from day 2 to 4 of the adaption period and on day 4 of adaptation baseline clinical health parameters were additionally taken.

Then ZEN treatment was administered from day 1. The diet then consisted of TMR and ZEN treatment bags which were manually added to the ration of each cow twice a day (7:00 a.m and 2:00 p.m.). Each bag of ZEN treatment consisted of 250 g ground wheat plus 4.6 mg ZEN, for a daily total of 9.2 mg ZEN per cow. To make sure that every cow would eat all of its treatment at every feeding event, only small portions of feed individually adapted to each cow's amount of intake and mixed with the treatment were offered at first (2-5 kg of feed). After the animals had finished this first portion, the rest of the ration was added.

During the treatment period of 21 days, clinical health parameters and chewing activity were measured on selected days, once a week (**Table 1**). After day 21 a washout period of 7 days followed, cows were weighted again (BW 598,57±171,02 kg) and they were brought back with the rest of the herd.

Throughout the experiment, the environmental temperature was monitored daily through two sensors installed inside the barn (PAPAGO measuring module, Papouch, Czech Republic).

Table 1. Schematic representation of the trial timeline. The colored cells per each column represent the days in which every measurement was taken. The specification "morning/evening" is added for measurements taken twice daily.

	Timeline	Weighing of cows	Clinical parameters	Chewing activity	Ultrasound
Adaptation	*start adaptation*	beginning			
	Adaptation day 2				
	Adaptation day 3				
	Adaptation day 4		morning/evening		
	Adaptation day 5				
Week 1	Treatment day 1				
	Treatment day 2		morning/evening		
	Treatment day 3				
	Treatment day 4		morning/evening		
	Treatment day 5				
	Treatment day 6				
	Treatment day 7				
Week 2	Treatment day 8				
	Treatment day 9		morning/evening		
	Treatment day 10				
	Treatment day 11				
	Treatment day 12		morning/evening		
	Treatment day 13				
	Treatment day 14				
Week 3	Treatment day 15				
	Treatment day 16		morning/evening		
	Treatment day 17				
	Treatment day 18		morning/evening		
	Treatment day 19				
	Treatment day 20				
	Treatment day 21				
Washout	washout				
	washout				
	washout				
	washout				
	washout				
	washout				
	all cows with the herd	end			

2.2 Animal clinical health parameter measurement

Clinical health parameters were measured on day 4 of adaption period and on days 2, 4, 9, 12, 16 and 18 of ZEN treatment period twice a day, always 1 hour after feed intake (9:00 a.m. and

4:00 p.m.). Body temperature was measured rectally with a thermometer (VT 1831, microLife, Switzerland). Heart rate was determined by palpating the *Arteria caudalis mediana* and counting pulse for 1 minute. Respiratory rate was visually collected by observing the right thorax for 1 minute. For ruminal peristalsis a stethoscope was used (Littmann Classic III, 3M, Germany), rumen was auscultated on the left side for 5 minutes to count the occurrence of physiological ruminal cycles in 5 minutes. Fecal consistency was evaluated through fecal consistency score (scale 1-4, with 1 being very soft and 4 being very dry and firm). Therefore, the cows were observed during measurement time and fecal score was evaluated visually right after defecation to ensure the right allocation. Heart rate, respiratory rate, rumen peristalsis and fecal consistency were all determined by the same person to obtain constant subjective observations. Every parameter was written down on papers right after evaluation and was then transferred to an Excel file.

2.3 Feeding behavior

For feeding behavior evaluation rumination halters were used (RumiWatch System, ITIN + Hoch GmbH, Liestal, Switzerland) witheach cow was wearing its individual halter (**Figure 1-3**). The halters were controlled and calibrated at the beginning of the trial and then were put on the cows. They stayed on the cows during days 2-4 of adaption period and during days 2-4, 8-10 and 16-18 of ZEN treatment period, measuring the following parameters:

- rumination time: time in minutes per day spent on rumination
- eating time: time in minutes per day spent on eating
- eating down time: time in minutes per day spent on eating with lowered head
- eating up time: time in minutes per day spent on eating with lifted head
- drinking time: time in minutes per day spent on drinking
- drinking gulps: amount of gulps per day
- ruminate chews per minute: amount of ruminate chews per minute
- ruminate chews per bolus: amount of ruminate chews per bolus
- chewing index: calculated as total chewing time divided by DM intake
- total chewing time: calculated as the sum of rumination time and eating time

The data were collected for 3 consecutive days per each experimental week. Every time the halters were removed after the selected days, data was converted and saved on a computer. Therefore, each RumiWatch had to be disassembled to get the access to the storage disk which then was connected with the laptop to collect the data. Data were downloaded and converted through software RumiWatch Manager and RumiWatch Converter.

Figure 1: Cow during measurements, wearing the RumiWatch halter (picture by Raul Rivera Chacon).

Figures 2 and 3: inside of the RumiWatch (RumiWatch System, ITIN + Hoch GmbH, Liestal, Switzerland), showing the battery and the data logger with the SD card (pictures by Raul Rivera Chacon).

2.4 Evaluation of the reproductive parameters

To evaluate the effects of ZEN on the reproductive parameters, rectal palpation and ultrasound evaluations of the uterus and ovaries were performed. For this part of the project, 7 cows were selected, namely the Brown Swiss from our research and other 6 animals out of the mentioned larger trial. The experimental setup for these animals was the same as previously described. The cows for the reproductive evaluations were selected together with the barn staff based on the pregnancy status and other aspects related to the management of the barn.

This project was part of a larger animal trial involving 18 animals. The 6 cows selected for this experiment were of different ages

Reproductive parameters were measured on day 1, 4, 10, and 17 of the treatment period. Therefore, reproductive trace was at first palpated manually: the uterus was checked for size, contractility, asymmetry and signs of pregnancy (asymmetry, cotyledons); for ovaries, size and occurrence of functional bodies was investigated. Afterwards an ultrasound (5-MHz linear-array transducer; Easi-Scan, BCF Technology Ltd., Bellshill, Scotland) was used for further investigation of the functional bodies on the ovaries (corpus luteum, follicle) (**Figure 4**).

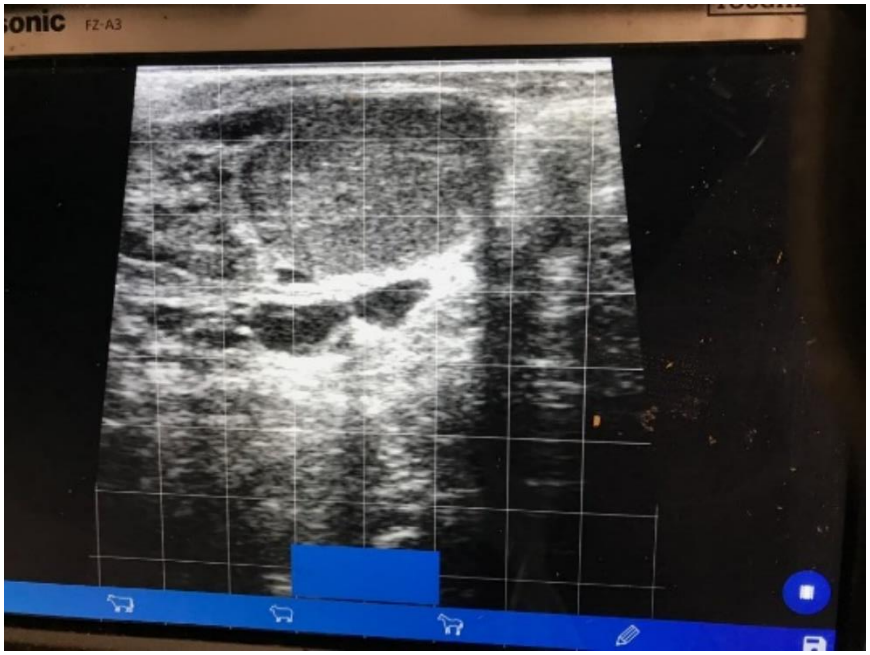

Figure 4: ultrasound image showing the right ovary of a cow with a corpus luteum that was palpated before. (picture by Rita Mühleder)

2.5 Statistical analyses

For statistical analysis of the data of clinical health parameters and chewing activity R statistical software (R Core Team, Austria) was used. The adaptation measurements served as baseline. The clinical parameters measurements collected during the ZEN treatment period were grouped in week 1 (days 2, 4), week 2 (days 9, 12), and week 3 (days 16, 18). Mixed linear models were applied for each response variable (like the aforementioned clinical parameters and chewing data) to consider both fixed effects (week, time of the day), and random effects, like cow-related characteristics (individual animal, parity, lactation) and environmental influences (environmental temperature):

$Y=a*X+b$

where Y is the dependent variable predicted by a linear effect of independent variable X with regression slope parameter or intercept a and the error term or random effect b. Mean value, estimate, standard error (*SE*), degrees of freedom (*DF)*, t-ratio and p-value were determined for pairwise comparisons between weeks and times of the day (a.m.-p.m.). P-values were adjusted for multiple testing with Benjamini-Hochberg method (47). As significance threshold of $P \leq 0.05$ was selected and trends were defined as $0.05 \leq P \leq 0.10$.

3 Results

3.1 Animal clinical health parameters

Measurement results are presented in detail in **table 2** and **figures 5-7**.

Body temperature increased from baseline measurement until week 2 ($P<0.05$) and then decreased again ($P>0.10$ for week effect). It showed to be slightly higher in the afternoon, but not significantly ($P>0.10$).

Heart rate decreased from baseline to week 1 ($P<0.05$), increased in week 2 ($P<0.05$) and decreased again in week 3 ($P<0.05$ for week effect). Heart rate was found to be higher during

the afternoon measurements than during the morning (P<0.05). This is also represented in **figure 5**.

Respiratory rate showed an increment from baseline to week 1, then decreased again in week 2 to almost the same mean value as for baseline measurement, then further decreased in week 3 (P>0.10 for week effect). Like heart rate, respiratory rate also showed to be higher in the afternoon (0.05<P<0.10).

Ruminal peristalsis showed higher mean values in week 1 than in baseline, then declined in week 2 (P<0.05) and remained under baseline value for the rest of the experiment (P<0.05 for week effect). Also ruminal peristalsis showed to be higher in the afternoon (0.05<P<0.10). This is shown in **figure 6**.

Fecal score mean value increased until week 2 to decline below baseline value in week 3 (P>0.10 for week effect). It showed to be higher in the afternoon (P<0.05), as shown in **figure 7**.

Table 2. Mean, standard deviation and P-values of measurements in the morning and afternoon per each week of the experiment. P-values are presented for the effect of time of the day (time), for the week and for the interaction between time of the day and week (Time × week).

	Baseline		Week1		Week2		Week3		P-Value		
Item	AM	PM	AM	PM	AM	PM	AM	PM	Time	Week	Time × week
Body temperature, °C	38.2 ±0.2 [a]	38.1 ±0.4 [a]	38.3 ±0.4	38.5 ±0.1	38.5 ±0.3 [b]	38.5 ±0.2 [b]	38.3 ±0.2	38.4 ±0.1	0.76	0.18	0.69
Heart rate, beats per minute	81 ±8 [a]	86 ±7 [a]	73 ±5 [b]	85 ±6 [b]	81 ±8 [a]	88 ±8 [a]	77 ±6 [a]	87 ±8 [a]	<0.01	0.01	0.27
Respiratory rate, breaths per minute	28 ±6	38 ±8	35 ±7 [a]	45 ±16 [a]	31 ±8	34 ±9	27 ±9 [b]	32 ±9 [b]	0.06	0.13	0.78

a, b = different superscripts indicate significant difference between weeks

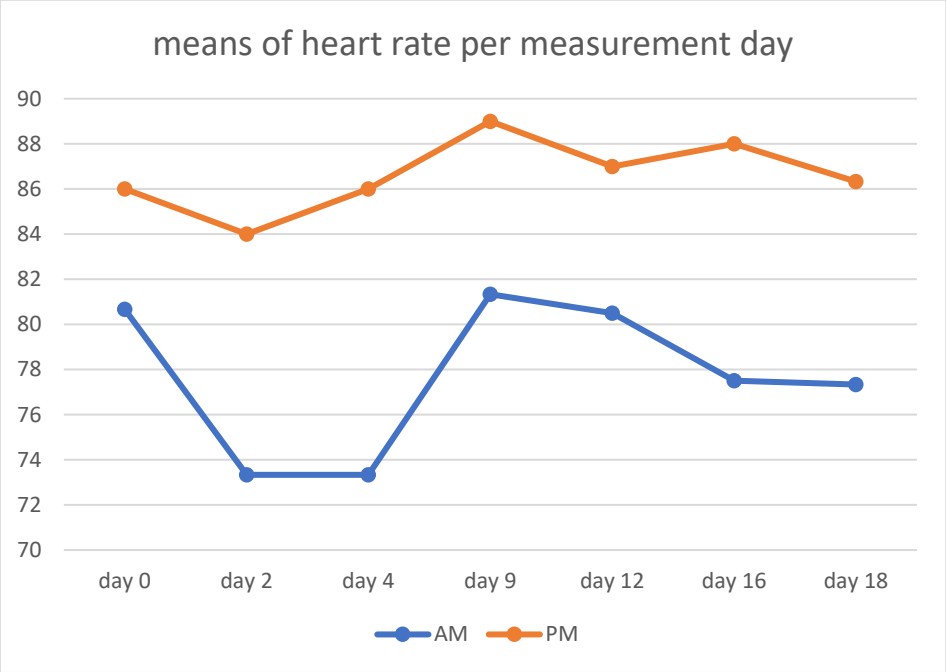

Figure 5. Mean values of heart rate per measurement day, morning (AM) and afternoon (PM) measurement for the six cows. y=beats per minute; day 0= baseline; day 2 & day 4 = week 1; day 9 & day 12 = week 2; day 16 & day 18 = week 3

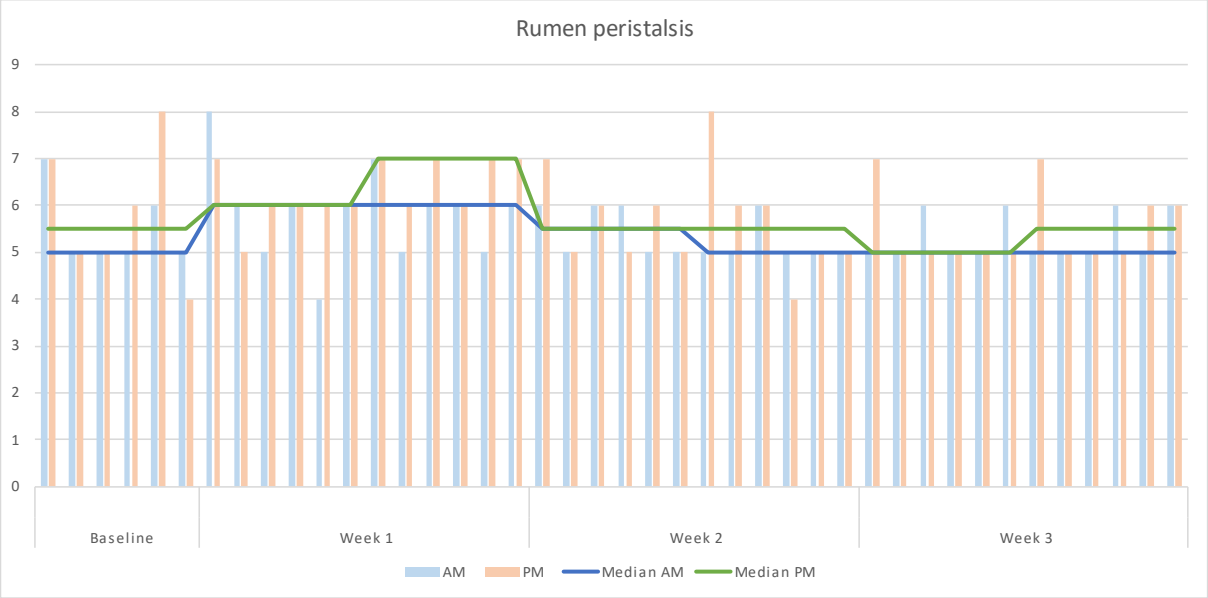

Figure 6. Values of rumen peristalsis per experimental week, morning (AM) and afternoon (PM) measurement for the six cows. Each bar represents the value for one animal. The lines represent the median over the experimental days. y= contractions per minute

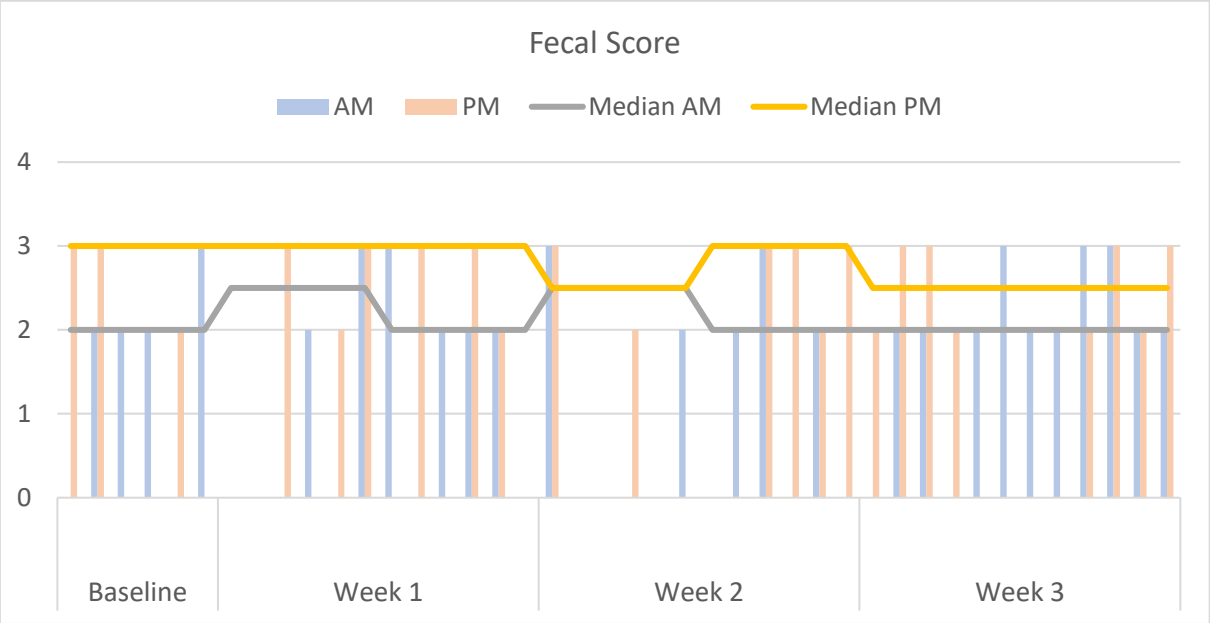

Figure 7. Values of fecal score per experimental week, morning (AM) and afternoon (PM) measurement for the six cows. Each bar represents the value for one animal. The lines represent the median over the experimental days. y=fecal score (scale 1-4, with 1 being very soft and 4 being very dry and firm)

3.2 Feeding behavior

No effects were observed on neither parameter of the feeding behavior. As already mentioned, parameters were measured each week for 3 continuous days and afterwards the mean values per each parameter was calculated for each day. In **table 3** the mean values for each week of all cows can be seen.

Table 3: mean, standard deviation and P-value of RumiWatch (RumiWatch System, ITIN + Hoch GmbH, Liestal, Switzerland) measurements per each week of the experiment. P-value is presented for the week effect.

Item	Baseline	Week1	Week2	Week3	P-value Week
Dry matter intake, kg	23 ±5	23 ±4	24 ±3	23 ±3	0.78
Rumination time, minutes	443 ±71	395 ±76	428 ±72	409 ±72	0.52
Eating time, minutes	172 ±52	178 ±68	170 ±54	179 ±48	0.83
Eating down time, minutes	103 ±45	102 ±57	89 ±38	89 ±32	0.60
Eating up time, minutes	70 [a] ±44	76 ±57	81 ±45	97 [b] ±50	0.15
Drinking time, minutes	10 ±6	10 ±6	11 ±5	9 ±7	0.58
drinking gulps, amount	141 ±79	133 ±81	148 ±72	145 ±94	0.58
Ruminate chews, per minute	68 [a] ±5	69 ±5	69 ±4	70 [b] ±5	0.17
Ruminate chews, per bolus	61 ±5	59 ±7	59 ±8	55 ±14	0.45
Chewing index	28 ±7	25 ±6	25 ±5	26 ±5	0.79
Total chewing time, minutes	615 ±111	573 ±112	598 ±108	588 ±82	0.86

a, b = different superscripts indicate significant difference between weeks

3.3 Reproductive parameters

No significant effects were observed on reproductive trace. Significant changes in size, contractility and symmetry of uterus were not found, neither in size ovaries nor functional bodies. In **table 4**, notes taken during rectal palpation are shown.

Table 4: rectal palpated findings; abberviations as follows:

U=uterus, LO=left ovary, RO= right ovary;

uterus: G=size, I=1 finger thickness, II=2 fingers, III=3-4 fingers, IV=as big as a bread, V=bigger than a bread and not all of it is palpable, VI=uteus is not palpable with one hand; K=contractility, I=nearly not contracted/slack, II=moderately contracted, III=strongly contracted (heat); S=symmetric, AS+ asymmetric.

Ovaries (size): eb=pea, bo=bean, ha=hazelnut, T=pigeonegg, W=walnut, H=chickenegg, E=duck egg, G=goose eg. GK=corpus luteum, F=follicle, FK=any functional body.

written in ()=ovary also found in the ultrasound→GK=corpus luteum, f=1 small follicle, fs=several small follicles, grube=follicular cavity, without=without any functional body.

Empty fields: ovaries not found on that day.

	cow 1, pregnant since 27.12.22			**cow 2,** non pregnant			**cow 3,** pregnant since 31.1.23			**cow 4,** non pregnant			**cow 5,** non pregnant			**cow 6,** pregnant since 30.11.22			**cow 7,** pregnant since 28.12.22		
day (date)	**U**	**LO**	**RO**	**U**	**LO**	**RO**	**U**	**LO**	**RO**	**U**	**LO**	**RO**	**U**	**LO**	**RO**	**U**	**LO**	**RO**	**U**	**LO**	**RO**
20.3.	GIII KII As+	W +GK		GII KII S	ha	ha +F	GII KII S	ha	T +GK	GII KII S	eb	T +F (1cm)	GII KII S	ha	H+FK	GV KI S	W		GII KII As+		W +FK
24.3.	GIV KI S	T	W +FK (GK)	GVI KI	eb	Eb	GV KI AS	eb	H +FK (GK, fs)	GII KII S	bo	T +grube	GII KII S	eb	W + FK (fs)	GV KI As+	T (fs)	W +FK (GK, f)	GIII KII S	W +FK (GK, f)	bo
30.3.	GIV KI S	W +F	T+ +FK (GK)	GVI KI S	eb	eb	GIII KI AS+	ha	H +FK (GK, fs)	GII KII S	eb	T +FK	GII KIII S	eb	W + FK	GIV KI S	ha	T +FK (GK +fs)	GI KI S	T +FK	ha
6.4.	GV KII S	T	W +FK (GK)	GVI KI S	Eb	eb	GIII KII AS+	bo	W +GK	GII KII S	eb	T without	GIII KI S	bo	H+FK	GV KI S	ha	W +GK	GII KII S	T +FK	ha

4 Discussion

The aim of our study was to research on effects of prolonged low dosed ZEN exposure on dairy cows clinical health parameter. Due to the low dosage used we did not expect any changes of the cows health parameters.

As the TMR itself was contaminated with minimal concentrations of ZEN only, observable effects can be led back to the experimental ZEN treatment. Furthermore, random effects, like daytime temperature and diurnal patterns in the animal, were taken into account in our statistical analyses. This is important, as clinical parameters might physiologically undergo daily variations – regardless of ZEN –, for example increasing daytime temperature, heat and humidity could increase body temperature and respiratory rate and reduce feed intake (48).

Our hypothesis, that the dosage of ZEN would be too low to affect the cows health, was rejected, as there were indeed unexpected effects on some clinical health parameters of the cows.

Changes in body temperature related to ZEN exposure were found in previous studies. Hartinger et al (38) explain the rising body temperature after short-term exposure to high dosage of ZEN as a typical immunological reaction to intoxication. Although an overall effect of ZEN on body temperature was not observed in our case, body temperature was significantly higher in week 2 compared to the baseline. The meaning of this result needs to be interpreted with caution, as on the one hand the deviations are rather small. On the other hand, this effect could be led back to animal specific reactions, though individual animal variation was taken into account by the model as random effect. Nonetheless, it is important to highlight that also for other parameters the pattern of changing values until week 2 before returning back to baseline was observed, which will be discussed later.

Overall effects on heart rate, respiratory rate, rumen peristalsis and fecal consistency were found.

The strongest effects were found on heart rate, with highly significant differences between daytime measurements. The cows showed higher values in the afternoon than in the morning. Of course, as previously mentioned, heart rate can change due to other influences, like daytime temperature or normal daily metabolic activity. However, since environmental temperature was included in our model as random factor, this effect can be also ascribed to effects of ZEN. A

possible explanation for this outcome could be the following: heart rate and energy expenditure show to be connected in ruminants as an increasing heart rate can be a reaction to a higher energy expenditure. This means that changes in the heart rate of an animal can be an indicator for changes in the energy status (49). The resting energy expenditure increases during the day, reaching its maximum in the afternoon (50). It is possible that the additional burden, namely ZEN exposure, brought energy expenditure so far that the heart rate changed. Also values of respiratory rate showed to be higher in the afternoon, which could also be explained by the increased energy expenditure due to stress caused by ZEN intake.

Furthermore, the heart rate did also change significantly between weeks. As we did not find any study with similar setups researching on effects on the heart rate, we can only compare this finding with the results from studies with short-term ZEN exposure, like in Hartinger et al (38). In this study, no effects on heart rate were found, which differentiates with our results. Still they mention that the heart rate can increase when the animals body is fighting with something, like ZEN in our case. This is also supported by other studies showing that changes in heart rate have been associated to sickness and chronic stress in dairy cows (51, 52). Yoshida et al (52) found higher heart rates in hospitalised cows (diagnosis: right abomasal displacement, hepatic dysfunction, chronic mastitis) than in the clinically healthy control group. Of couse the higher heart rates could also be a reaction to pain in this case and comparisons should be made with caution. Still their findings could also be reactions to chronic stress, which would be ZEN intoxication in our case. The differences to the study by Hartinger et al (38), where overall changes in body temperature were found, can be explained by the fact that they rather focused on the acute response (hours after exposure) while we evaluated the prolonged exposure.

Concerning intestinal trace, we found significant changes in ruminal peristalsis. The frequency of rumen cycles per 5 minutes showed the trend to be slightly higher in the afternoon, but throughout decreased during the project (baseline to week 3). This decrease might be led back to the influence of ZEN on ruminal microbiome composition and activity. As part of our study we could not arrange to evaluate those parameters, but other studies did research on them. Our experimental setups could at best be compared with results of the research by Hartinger et al (38), who investigated effects of ZEN after short-term exposure by feeding six cows 5 mg of ZEN per day. As previously stated, they found rumen pH and SCFA to decrease, as well as

essential microorganisms, like *Lachnospiraceae* and *Prevotellaceae*. Commonly known, a decreasing pH (found for example in ruminal acidosis) will also influence digestion and ruminal peristalsis negatively (53). Therefore, our results seem to be plausible, although it would be important to collect data on microbial activity in the rumen in response to prolonged ZEN exposure. Furthermore, Deoxynivalenol, another toxin produced by *Fursarium* was found to reduce the contractility of smooth muscle cells and their proliferation and to promote intestinal dysmotility (54).

The fact that peristalsis increased in the afternoon does also fit to the increase of the other parameters: an increased passage rate reduces the metabolization capacity by the rumen (55). Therefore, the increased heart rate observed in parallel with increased peristalsis might indicate a reduced degradation of ZEN and a toxic effect.

The increasing fecal score in the afternoon, meaning slightly firmer feces, could again be caused by the previously mentioned energy expenditure, which would be higher due to loss of energy to ZEN degradation. The connection between fecal score and energy expenditure is also given in pathogenic mechanism of ketosis, where feces appear to be firmer (56).

A phenomenon could be observed in all the measured parameters: at the beginning of ZEN exposure there was an increase or at least an increasing trend, followed by a decrease back to baseline or even below baseline levels. For body temperature, heart rate and fecal score, values continuously went up until week 2 and then went back to baseline. For respiratory rate and peristalsis, rising values only lasted until week 1, for week 2 they were similar to baseline and then even went below baseline values. This might best be explained by a habituation effect of the cows to ZEN exposure. The reduced peristalsis by the end of experiment could represent a physiological adaptation of the rumen to facilitate the degradation of ZEN. This would also have an effect on the metabolism capacity of ZEN and, as a consequence, on the other health parameters. The question of what would happen when cows are exposed to ZEN contamination even longer than 3 weeks remains to be researched on in future studies.

Interestingly, none of the parameters measured by RumiWatches® was influenced by the ZEN exposure. The importance to use both measurements was given in order to collect more and different data and to have data measured on the on hand manually or in person and by a device

on the other hand. Since measuring constantly during 3 days a week, the RumiWatches® might provide more accurate and precise data (57). However, the absence of changes for the parameters measured by RumiWatches® does invalidate results of clinical parameters. Much more, they should represent an additional confirmation of possible changes. Ruminal peristalsis and fecal score, two parameters directly showing health condition of the animal, can still be regarded as highly important and meaningful.

It is not surprising that no significant alterations of the reproductive trace linked to the low dose ZEN exposure were found. There were alterations in uterus size and contractility, but since random effects like cycle phase and pregnancy were not taken into account, it cannot be stated that those variations are responses to ZEN exposure. It is much more likely that the expanding uterus found in the pregnant cows is the physiological growth of the fetus, and the consistent functional body of them is the pregnancy-preserving corpus luteum. Concerning the cyclic cows, uterus and ovaries undergo physiological changes of hormonal influence during estrus, metestrus, diestrus and proestrus (58). To gather more meaningful results about effects of low dosed ZEN exposure to reproductive trace, we recommend another study with more animals in the same or at least similar cycle phase or stage of pregnancy.

The detection of ZEN contamination in herds usually is a consequence of the observation of problematics related to reproductive parameters or performance (59). However, our study indicates that exposure to ZEN could be detectable earlier with clinical assessment of animal health.

The observed effects on general cows health represent an important finding due to the novel experimental design, which allowed to assess the effect of prolonged exposure to ZEN. Although traces can be found in the rumen up to 34 hours after intake, the majority of ZEN is metabolized within 24h (60). However, ZEN could accumulate in the rumen due to the prolonged exposure and could impair the ruminal metabolism in the long term, causing the effects we observed. For example, it has been previously reported that ZEN intake in concentrations below the recommended threshold can accumulate in the bile of cows (61).

Although the health parameters seemed to return to values comparable with our baseline measurements by the last week of experiment, it can’t be excluded that the gastrointestinal

metabolism and function could be impaired by chronic exposure to ZEN, with consequences on general animal health. Further studies are warranted to establish the possible correlation between the onset of clinical symptoms and the amount of ingested ZEN and its metabolites that might accumulate during prolonged exposure to contaminated feed.

5 Conclusion

This is one of few studies to confirm that even low amounts of ZEN can be harmful to dairy cows health when fed over a prolonged time period. This study highlights the relevance of this hazard for dairy cows health with contamination of feeds with ZEN and other mycotoxins,. Furthermore, it accentuates the urge for more researches on prolonged effects of low dosed mycotoxin contamination in dairy cattle, especially in context of general herd health and case history of appropriate symptoms. As fertility of livestock is of great economical interest, research on estrogenic effects of ZEN remains extremely relevant.

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