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Deoxynivalenol and Zearalenone Residues in Eggs of Laying Hens Fed with a Naturally Contaminated Diet: Effects on Egg Production and Estimation of Transmission Rates from Feed to Eggs

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The potential for the *Fusarium* mycotoxins 4-deoxynivalenol (DON) and zearalenone (ZON) to enter the human food chain through contaminated eggs was assessed using a controlled feed study. Four groups of laying hens (eight in each group) were fed a diet that included differing amounts of naturally contaminated wheat containing DON (≈20 mg kg^{-1}) and ZON (0.5 mg kg^{-1}). Eggs were collected and pooled from each group on a daily basis. Pooled samples were analyzed by liquid chromatography with mass spectrometry detection (LC-MS/MS). The method allowed DON, other type B trichothecenes, ZON, and its metabolites to be determined in a single multi-residue analysis. The selectivity of the MS/MS procedure allowed cleanup to be minimized (for DON, cleanup by immunoaffinity column was used) or eliminated (for ZON). The limits of detection of 0.01 μg kg^{-1} for DON and 0.1 μg kg^{-1} for ZON in eggs were lower than previously published methods. None of the samples analyzed had detectable levels of ZON or its metabolites. Although maximum levels of DON contamination (10 mg kg^{-1} feed) were relatively high, no adverse effects were observed on egg production. On the basis of the determined DON levels in the hen's diet and the determined levels of DON in the corresponding eggs, transmission rates of 15 000:1, 18 000:1, and 29 000:1 for treatment levels 5, 7.5, and 10 mg DON kg^{-1} feed, respectively, were found. These results show that, although eggs could be a human exposure route for DON, the levels are insignificant compared to the other sources, although the presence of metabolites of DON was not studied.

KEYWORDS: Deoxynivalenol; Zearalenone; *Fusarium* mycotoxins; LC-MS/MS method; eggs; laying hens; transmission rate

INTRODUCTION

Trichothecenes and zearalenone (see **Figure 1**) are secondary metabolites produced by some species of several fungal genera, most notably *Fusarium*. Their prevalence mainly depends on weather conditions during plants' growing period. *Fusarium* species are widespread in nature and commonly contaminate many cereal grains intended for human and animal consumption. As these mycotoxins are frequently found in cereals (wheat, corn, barley, oats, and rye), they are of concern from a food safety perspective regarding human exposure.

Trichothecene mycotoxins compose a large group of substances. At present, over 150 trichothecenes are known, although only 20 are naturally occurring and of interest concerning human exposure. Deoxynivalenol (trichothecene of type B) is mainly found in cereal samples and is often used as an indicator of Fusarium infection, although other trichothecenes such as nivalenol (NIV, trichothecene of type B), HT-2 toxin, and T-2 toxin (trichothecenes of type A) are also often present. 4-deoxynivalenol (DON), as well as other trichothecenes, exhibits toxicity at the sub-cellular, cellular, and organic system level largely due to its ability to inhibit protein synthesis and to covalently bond to sulfydryl groups. In animals, which are more likely to be exposed to higher dose levels of DON than are humans, the effects of exposure are mainly expressed as severe reduction in weight and impaired resistance to infection, particularly bacterial infection. Common symptoms of acute toxicity of DON are nausea, vomiting, dermal irritation and lesions, haemorrhagic lesions, and pathological changes in the haemopoietic organs (*1*). A provisional tolerable daily intake (TDI) of 1 μg/kg bw/day has been recommended by the Scientific Committee on Food (SCF) for DON (*2*).

Zearalenone (ZON), a derivative of β-resorcyclic acid lactone is a natural toxin produced by *Fusarium spp.* It mainly occurs in infected corn or wheat often together with trichothecene

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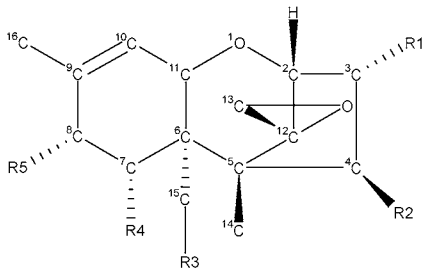
Trichothecene	R1	R2	R3	R4	R5
Type A					
T-2 toxin	OH	OAc	OAc	OH	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OAc	OH	$OCOCH_2CH(CH_3)_2$
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
Neosolaniol	OH	OAc	OAc	OH	OH
Type B					
Deoxynivalenol	OH	H	OH	OH	O
3-acetyl-deoxynivalenol	OAc	H	OH	OH	O
15-acetyl-deoxynivalenol	OH	H	OAc	OH	O
Nivalenol	OH	OH	OH	OH	O



Type A and Type B trichothecenes

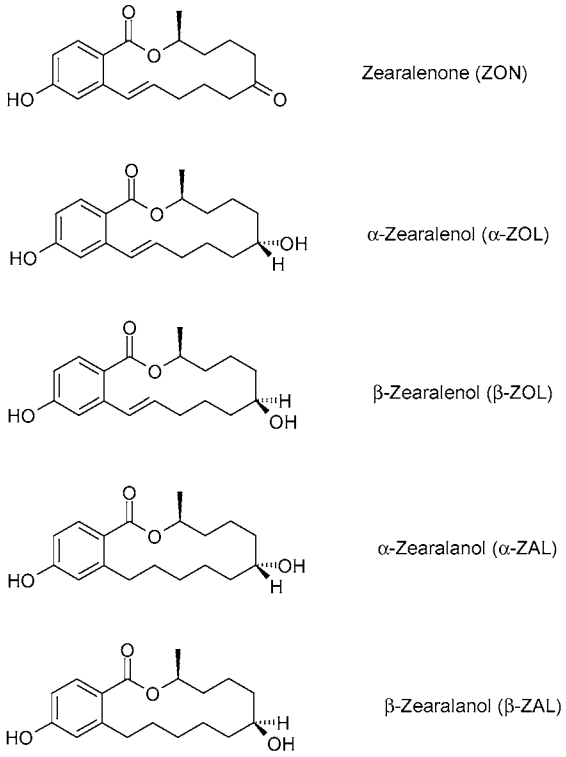


Figure 1. Chemical structures of deoxynivalenol and zearalenone.

mycotoxins (DON, NIV). Reproductive disturbances and alterations in genital organs that have been observed in swine and rodents (*3*) are related to the capability of ZON to bind to the oestrogen receptors. Analogues or metabolites of ZON, such as α-zearalenol (α-ZOL), *β*-zearalenol (*β*-ZOL), α-zearalanol (α-ZAL, zeranol) and *β*-zearalanol (*β*-ZAL, tarelanol) can be found naturally or as result of metabolism of ZON in animals (for example excreted to milk of lactating cows) or humans. Furthermore, ZON and its metabolites can also exist as conjugates, mainly as glucoronides or sulfates (*4*, *5*).

Level of trichothecenes and ZON and its metabolites in food and animal feed have been of concern within the European Union (EU). The European Commission has recommended that Member States take preventative measures aimed at reducing ZON levels in cereals and that monitoring for the presence of the Fusarium toxins should be continued especially in risk products such as infant foods (*6*). One route of exposure that may not have been considered in the past for *Fusarium* toxins is from animal feed, leading to residues in animal products such as eggs.

Poultry are capable of tolerating relatively high intakes of trichothecenes in their diet without exhibiting deleterious effects and therefore could be an inconspicuous route of human exposure via contaminated feed. There is minimal information on the occurrence of trichothecenes in eggs or rates of transmission. Prelusky et al. (*7*) investigated the possible transmission of DON metabolites to eggs with the aid of extensively enriched ^{14}C-DON by measuring radioactivity. They found that consumption of 550 μg of DON/day produced a maximum transmission level of 0.31% from feed to eggs. The same group reported 0.19% transmission of daily DON administered to the eggs, as bound DON and DON-metabolites (*8*). In fact, only 10% of this radioactivity could be extracted and measured as DON by GC-MS, suggesting the un-extracted portion was either conjugated (probably glucuronide conjugates) or present as metabolites. Trichothecenes are known to undergo either deacetylation (hydrolysis), hydroxylation (oxidation) or de-epoxidation (reduction) in the body (*8*). Another study found radioactivity in eggs after the administration of ^{3}H labeled T2 toxin up to approximately 0.17% of the administered dose (*9*). El-Banna et al. (*10*) fed hens with rations contaminated with 5 mg kg^{-1} DON for 192 days. No traces of DON were found in the tissues or eggs using a GC-MS method with detection limit of 10 μg DON kg^{-1}.

Unfortunately, information about ZON and its metabolites in eggs resulting from natural low-level exposure is still missing. It is speculated that prolonged exposure to ZON could lead to an accumulation of the toxin in the yolk of eggs due to the lipophilic nature of the toxin. The only published study used ^{14}C labeled ZON (*11*). Approximately 1% of the radioactivity was found in eggs up to 72 h after a single dose of labeled ZON. However, the compounds were not identified, and so it is unclear whether it was parent ZON or metabolites. However, analysis of urine and tissue samples has been widely reported in swine and cattle (*12*, *13*).

The carry-over of *Fusarium* mycotoxin residues from feed to animal tissues can be expressed as a ratio of *n*:1, where *n* is the concentration of the analyte in feed needed for recovery of 1 unit of concentration of the residue of analyte in the edible product. In the case of eggs of laying hens, many different average transmission ratios have been reported, but only for aflatoxins (e.g., for aflatoxin B_1 5000:1, 40000:1, or 2200:1) (*14*−*16*). There are no current data on residues of *Fusarium* mycotoxins in eggs resulting from natural exposure. The aim of the present work was to evaluate the transmission of residual *Fusarium* mycotoxins (DON, ZON) into the eggs of laying hens fed rations containing different levels of the toxins.

Gas chromatography with mass spectrometry detection (GC-MS) has been frequently applied to the determination of trichothecenes in a variety of cereal-based foodstuffs (*17*, *18*). Current GC-MS methods for cereals have detection limits of approximately 5−20 μg kg^{-1} for each trichothecene (a range

Table 1. Levels of DON in Feeds Administered to Laying Hens

		treatment level (mg kg^{-1})			
sub-sample	level found (mg kg^{-1})	group A 0 mg kg^{-1}	group B 10 mg kg^{-1}	group C 7,5 mg kg^{-1}	group D 5 mg kg^{-1}
I. (1st week)	17.8	0[a]	8.90	6.68	4.45
II. (2nd week)	19.5	0[a]	9.75	7.31	4.88
III. (3rd week)	19.2	0[a]	9.60	7.20	4.80
IV. (4th week-reacclimat.)		0[a]	0[a]	0[a]	0[a]

[a] Limit of determination of the analytical method (LC-MS/MS) for DON in wheat was 1.5 μg kg^{-1}.

of 8−10 analytes can be included in a single analysis). To improve these detection limits, the extract injected into the GC-MS would need to be highly concentrated, and samples usually need to undergo a multistep cleanup (MycoSep, immunoaffinity column, Florisil etc.) (*1*). Almost all GC methods for determination of trichothecenes are based on prior derivatization that can be time-consuming and prone to error. LC has been used with either photodiode array or fluorescence detection (after derivatization). The major disadvantage of LC is the extensive and/or very selective sample cleanup procedures required to remove matrix components that might interfere with the analyte signal(s) and the need to preconcentrate the analyte(s) to reach the required detection limits. Recently published LC methods using fluorescence detection for determination of ZON in cereals (with cleanup step by affinity column) show a reporting limit of 5 μg kg^{-1}. In contrast, on-line coupling of LC with MS/MS can reduce the need for sample preparation, since coeluted compounds may be eliminated by MS selectivity. As the method includes only a few, it allows for a high throughput of samples. Recent papers have demonstrated the great potential of LC-MS/MS for the simultaneous quantitative determination of a variety of mycotoxins, including trichothecenes (*19*−*21*). A further advantage of an LC-MS/MS approach is that it offers the opportunity to combine the determination of both trichothecenes and ZON and metabolites into a single multi-residue analysis.

MATERIALS AND METHODS

Experimental Design. A total of 32 19-week-old laying chickens (Bovan Goldline strain, which are derived from White Leghorns with addition of other genetic material) were randomly assigned into four experimental groups (8 birds in each). Birds were individually housed in sight of other birds and provided with nest boxes, perches, and items for environmental enrichment. Chickens were maintained for 3 weeks for acclimatization, and during this period they were fed with proprietary chicken layer ration (200 g/day) based on wheat, soya, and sunflower seeds, that had been previously analyzed for *Fusarium* mycotoxins (see **Table 1**), supplemented by oyster shell grit (50 g/day). The chickens were exposed to mycotoxins from naturally contaminated feed for 3 weeks and then fed again with proprietary chicken layer ration for 1 week before euthanasia by cervical dislocation. The dietary treatments were 0 (control), 5, 7.5, and 10 mg DON kg^{-1} feed together with 137.5, 206, and 275 μg of ZON kg^{-1} feed. Oyster shell grit was offered as before the trial. All eggs produced were recorded, collected, and individually weighed. Before homogenization and storage of eggs at −20°C, the weight of shell and content of egg (yolk together with white) were recorded. Feed and water were provided ad libidum before the trial. During the trial, feed and water were provided in measured quantities that were in excess of the birds requirements. Feed and water consumption were recorded individually on a daily basis, body weight was determined weekly for each bird.

Preparation of Contaminated Rations. Naturally contaminated wheat was produced by artificially inoculating grain (in middle of anthesis) with a conidial suspension containing spores from 5 *Fusarium culmorum* isolates (all of them DON producers) and 5 *F. graminearum* isolates (2 of them NIV producers). A 12-kg sample of contaminated wheat (containing 19.8 mg DON kg^{-1} and 0.55 mg ZON kg^{-1}) was homogenized in a Tumble mixer and divided into three 4-kg subsamples (I., II., III.). These were mixed in different proportions with the proprietary chicken layer ration. Concentrations of DON (**Table 1**) were confirmed by analyzing 0.5-kg samples from each portion using the LC-MS/MS method described. Additionally, 3-acetyldeoxynivalenol (3-AcDON) and 15-acetyldeoxynivalenol (15-AcDON) were detected in wheat samples using GC-MS, but the levels were considered to be insignificant (0.14 and 0.04 mg kg^{-1}, respectively). Feed was separately prepared for each bird in each group for the 21 days of the trial, see **Table 1**.

Table 2. Electrospray Negative Ionization Mode MS/MS Conditions for Target Analytes

analyte	retention time (min)	precursor ion mass (*m*/*z*)	production mass (*m*/*z*)	collision energy (eV)	cone voltage (V)
nivalenol	5.6	370.8	310.8	10.0	50.0
		310.8	281.0	10.0	50.0
deoxynivalenol	**7.1**	**294.9**	**265.0**, **137.9**	**15.0**	**50.0**
β-zearalanol	9.4	320.9	303.0, 277.0	20.0, 23.0	60.0
α-zearalanol	9.8	320.9	303.0, 277.0	20.0, 23.0	60.0
β-zearalenol	9.6	318.9	275.0, 188.0	23.0, 30.0	60.0
α-zearalenol	9.9	318.9	275.0, 188.0	23.0, 30.0	60.0
zearalenone	**10.2**	**316.8**	**273.0**, **175.0**	**20.0**, **25.0**	**60.0**

Analysis of Trichothecenes and ZON in Wheat. (*A*) *Trichothecenes.* At the beginning of the study, for the screening of naturally contaminated wheat, a multi-residual GC-MS method was employed which is routinely used for determination of trichothecenes in grains. The GC-MS method, which can determine nine trichothecene mycotoxins (DON, NIV, 15-AcDON, 3-AcDON, diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), neosolaniol (NEO), HT-2 toxin (HT-2 tox.), and T-2 toxin (T-2 tox.)) in wheat was based on a published method by Radova et al. (*22*). For the cleanup of a crude acetonitrile−water (84:16, v/v) extract, solid-phase extraction (SPE) employing MycoSep225 column was used. Volatilisation of analytes prior to the final determinative step was performed by trifluoroacetic acid anhydride (TFAA) derivatization. Trifluoroacyl derivatives of the trichothecenes were separated by high-resolution capillary gas chromatography employing mass spectrometric detection. The detection limits for target analytes ranged from 5 to 100 μg kg^{-1}, depending on the chemical characteristics of the particular toxin (number of derivatized hydroxyl groups).

(*B*) *Deoxynivalenol.* For determination of DON in wheat sub-samples (I., II., III.) and proprietary chicken layer ration, a multi-residue LC-MS/MS method was used. Wheat samples were extracted with acetonitrile−water (84:16, v/v) by shaking for 2 h. After centrifugation and filtration, an aliquot of 500 μL of crude extract was diluted with 500 μL of methanol−water (2:8, v/v), filtered through micro-filter (4-μm pores) and analyzed by LC-MS/MS (described below). The limit of detection of the analytical method for DON in wheat was 1.5 μg kg^{-1}. Recoveries at spiking levels of 20 μg DON kg^{-1} and 150 μg DON kg^{-1} were 94.4 ± 3.9% and 84.1 ± 11.7% (n = 3), respectively.

(*C*) *Zearalenone.* Extraction and quantification followed the method published by Schuhmacher et al. (*23*). Homogenized wheat samples were extracted with acetonitrile−water (75:25, v/v) by using Ultra-Turrax for 3 min. To an aliquot of extract (12 mL) Phosphate Buffered

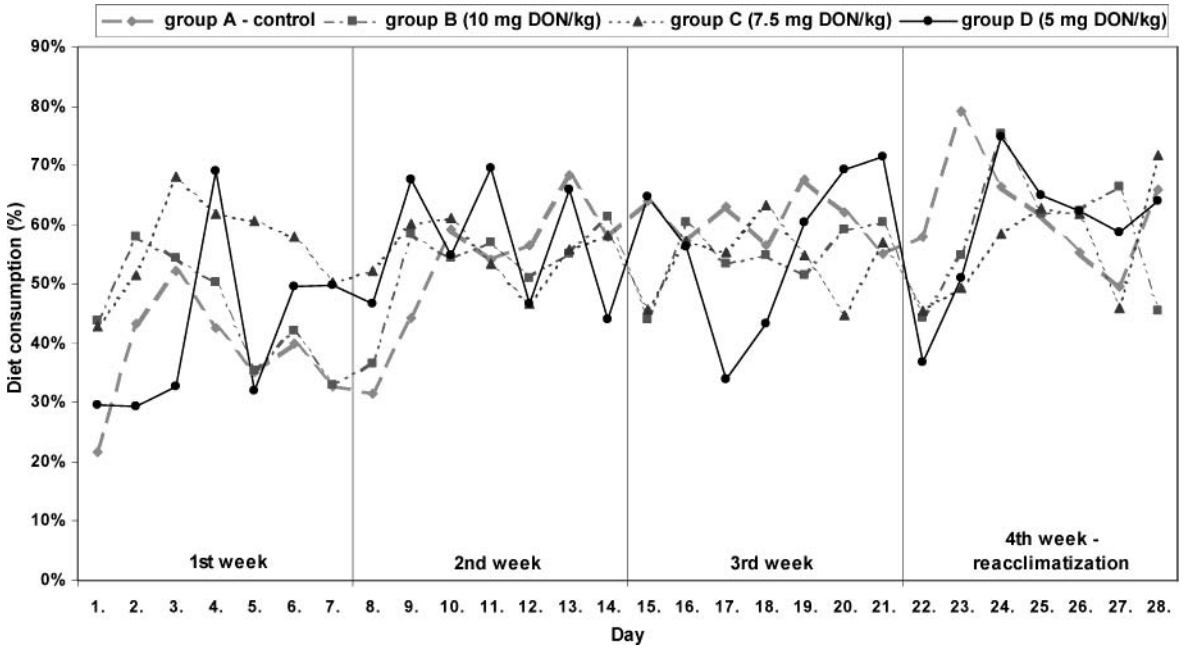


Figure 2. Average feed consumption per day in the four groups with different treatment levels (0, 5, 7.5, and 10 mg DON kg^{-1}).

Table 3. Daily Egg Production and Egg Weight during the 21-day Exposure of Laying Hens to Different Levels of DON

	egg production (%)[a]				egg weight (g)[b]			
	group A **0 mg kg^{-1}**	group B **10 mg kg^{-1}**	group C **7.5 mg kg^{-1}**	group D **5 mg kg^{-1}**	group A **0 mg kg^{-1}**	group B **10 mg kg^{-1}**	group C **7.5 mg kg^{-1}**	group D **5 mg kg^{-1}**
I. (1st week)	94.6 ± 7.1	100.0 ± 0.0	91.1 ± 10.4	82.1 ± 12.0	57.6 ± 3.9	57.4 ± 3.5	52.3 ± 4.5	55.2 ± 5.3
II. (2nd week)	96.4 ± 9.8	89.3 ± 18.8	87.5 ± 23.3	83.9 ± 20.6	57.3 ±3.3	54.8 ± 4.2	50.3 ± 5.7	53.0 ± 3.2
III. (3rd week)	87.5 ± 8.2	78.6 ± 12.0	80.4 ± 19.8	78.6 ± 25.5	59.3 ± 3.4	52.7 ± 4.5	50.2 ± 3.6	51.9 ± 7.7
IV. (4th week-reacclimat.)	83.9 ± 7.3	83.9 ± 11.3	76.8 ± 19.8	85.7 ± 13.1	60.9 ± 9.4	52.5 ± 4.2	50.1 ± 3.8	54.6 ± 3.0

[a] Mean ± RSD of eight birds in each group according to daily production (maximal production in each group was 8 eggs per 8 birds). [b] Mean ± RSD of weekly production in each group.

Table 4. Feed Consumption and Mycotoxin Ingestion during the 21-day Exposure of Laying Hens to Different Levels of Contaminated Diet

	feed consumption (g/bird/day)[a,b]			
	group A **0 mg kg^{-1}**	group B **10 mg kg^{-1}**	group C **7.5 mg kg^{-1}**	group D **5 mg kg^{-1}**
I. (1st week)	97.9 ± 20.8	122.4 ± 17.4	95.1 ± 16.8	83.6 ± 23.1
II. (2nd week)	97.7 ± 19.7	123.,4 ± 17.4	108.9 ± 9.8	97.2 ± 16.0
III. (3rd week)	112.0 ± 13.6	108.5 ± 9.5	101.7 ± 5.6	107.4 ± 21.6
IV. (4th week-reacclimat.)	109.2 ± 17.8	118.0 ± 21.3	99.6 ± 22.9	117.5 ± 18.9
avg I.–III. week	101.2 ± 18.3	105.9 ± 12.5	102.7 ± 10.2	99.0 ± 19.2
avg DON ingestion (mg/bird/day)[c]	**0**	**1.11 ± 0.08**	**0.72 ± 0.,08**	**0.44 ± 0.07**
avg ZON ingestion (μg/bird/day)[c]	0	35.2 ± 2.3	21.0 ± 1.4	13.2 ± 1.6

[a] Daily rations were 200 g/bird [b] Mean ± RSD of eight birds in each group [c] Estimated considering the actual concentration of DON in the rations and average feed consumed in each group per week.

Saline (88 mL) was added and cleaned-up by automated immunoaffinity column (IAC). Quantification was carried out using high performance liquid chromatography with fluorescence detection (HPLC-FLD, ex/em 275/450 nm). The limit of detection for target analyte was 3 μg kg^{-1}. Recovery of the analytical method for ZON (spiking level 50 μg kg^{-1}) was 85.0 ± 7.5% (n = 3).

Analysis of DON and ZON Together with its Metabolites in Eggs. For each group, daily egg production was pooled (albumen plus egg yolk) in a blender jar and gently blended for 1 min, with the exception of two eggs from the same hens, which were stored separately (one as mixed albumen and yolk, one as yolk separate from albumen), which were available for use in further experiments. The pooled homogenized samples (4−6 per day) were stored in a freezer (−20 °C) prior to analysis.

For analysis, a 20-g aliquot of the pooled sample was extracted with 50 mL of acetonitrile by shaking for 2 h. This was then centrifuged (15 min/4000 rpm) and filtered. The crude extract was divided into two portions for determination of target analytes, one for the determination of DON and the other for the determination of ZON, α-ZOL, β-ZOL, α-ZAL, and β-ZAL. For DON analysis, a 3-mL aliquot was evaporated to dryness, redissolved in 1 mL of water, followed by cleanup using immunoaffinity column (elution 1 mL MeOH, DON-

IACH-Vicam). The cleaned fraction was evaporated to dryness, redissolved in 500 μL of MeOH-water (2:8, v/v), and analyzed by LC-MS/MS. For the determination of other target analytes (ZON and its metabolites), 500 μL of crude extract was diluted with 500 μL of MeOH−water (2:8, v/v) and directly analyzed by LC-MS/MS.

Identification and quantification was achieved using a Waters (Milford, MA) Alliance 2695 LC system equipped with Micromass (Manchester, UK) Quattro Ultima tandem mass spectrometer. The mass spectrometer was operated in the negative electrospray ionization mode at a capillary voltage of 3.0 kV, a desolvation temperature of 350 °C, source temperature 120 °C and a cone voltage of 50 V (ZON and its metabolites 60 V). Desolvation gas was nitrogen, and CID gas was argon. For each analyte, two highly abundant product ions were monitored using the conditions given in **Table 2** (SIM mode). The analytical column was a 150- × 2.1-mm i.d. 4-μm C_{18} Jones Chromatography (Crawford Scientific, UK), thermostated at 30 °C. Gradient mobile phases consisted of (A) water and (B) methanol at a flow rate 0.2 mL min^{-1}. Linear gradient was started at 0 min (90% A) toward 10% A at 3 min and kept at that composition until 6 min, followed by linear gradient toward 90% A at 10 min and kept at that composition until 15 min.

RESULTS AND DISCUSSION

No overt changes that might be associated with toxin consumption were observed in the general state of the laying hens during the experimental period. Health status was unchanged. No stereotypical behaviors were noted, and none of the birds included in the study were discarded. Average increase in body weights differed between groups during the 3-week period. When normalized data were used to obviate the effect of different starting weights, significant differences ($p < 0.05$) were seen between group A and groups B and C for weeks 2 and 3. A significant difference ($p < 0.05$) was also observed between group C and D on the second and third week. The order of increase in mean body weight was found to be A > D> B > C after the normalization process. While the control group (A) and the group fed with lowest treatment level (5 mg DON kg^{-1}) (D) showed an increase in body weight (3.4 and 1.8%, respectively) during the 3-week period, the other two groups (B and C), with higher treatment levels of DON, showed a decrease in body weight (5−6%). A comparison of the feed consumption between tested groups is shown in **Figure 2**. Feed consumption was not affected by dietary treatments (average diet consumption was 54% of daily dose, $p > 0.05$). Average DON ingestion was 0.44, 0.72, and 1.11 mg/bird/day for treatments of 5, 7.5, and 10 mg kg^{-1} feed respectively, as estimated by considering actual concentration of DON in rations fed to laying hens and average feed consumed in each group during the 3-week period. Nevertheless, some dietary preferences were observed between hens from different groups. Each portion of the hens' diet was a mixture of proprietary chicken layer and contaminated wheat, and hens were fed every day at the same time. Observations were made by technicians who took care of animals during the study that birds from the group with the lowest treatment level preferred to eat the contaminated wheat first, birds from the middle treatment group preferred to eat the proprietary chicken layer first, and birds from the highest treatment group ate ration without any obvious preference. Every day for each hen at the same time, the rest of the diet was weighed and water consumption was recorded. As the feed was ad libidum in all cases, the calculated DON ingestion can therefore be taken only as an approximation.

The results of feeding DON-contaminated diets on average weekly egg production, egg weight, feed consumption, and approximate DON ingestion of laying hens over the 28-day period are shown in **Tables 3 and 4**. Thin or soft-shelled eggs are a common phenomenon after stress application. It is not uncommon for its effects to persist in the eggs' shell for a period of 20 days (*24*). One of the first symptoms of changes and health problems for laying chickens is cessation of egg production as the hens fed with contaminated ration may not have had sufficient nutrients in their diet. Weekly egg production was similar for all groups with the lowest production in the third week of feeding with contaminated rations (**Table 3**). After stopping the contaminated ration feed in week 4, the production of eggs increased for the highest (group B) and the lowest (group D) treatment levels, although surprisingly, in the middle-treatment level (group C) and the control group, egg production continued to drop. In contrast to the literature, the thickness of the shell showed a mounting trend with increasing dose of contaminated diet. After three weeks of consumption of contaminated rations, average shell weight had risen by 2.8, 2.9, 10.1, and 12.3% in the control group, lowest dose (5 mg DON kg^{-1}), highest dose (10 mg DON kg^{-1}), and middle dose (7.5 mg DON kg^{-1}) groups, respectively.

For determination of target *Fusarium* mycotoxins (DON and ZON and its metabolites) a multi-residue LC-MS/MS method was employed using a separate immunoaffinity column cleanup for the determination of DON. Repeatability was tested for all

Table 5. Residues of DON (μg kg^{-1}) in Eggs during the 21-day Exposure of Laying Hens to Different Levels of DON and the 8 days of Reacclimatization Period[a]

		treatment level			
day of treatment		group A control 0 mg kg^{-}	group B 10 mg kg^{-1}	group C 7.5 mg kg^{-1}	group D 5 mg kg^{-1}
1st week	1	< LOD	< LOD	< LOD	< LOD
	2	< LOD	0.13	0.19	0.15
	3	< LOD	0.22	0.21	0.49
	4	< LOD	0.24	0.28	0.28
	5	< LOD	0.54	0.47	0.29
	6	< LOD	0.62	0.43	0.44
	7	< LOD	0.47	0.63	0.30
1st week avg		< LOD	***0.37*** ± 0.20	***0.37*** ± 0.17	***0.33*** ± *0.12*
2nd week	8	< LOD	0.38	0.79	0.47
	9	< LOD	0.23	0.74	0.32
	10	< LOD	0.26	0.75	0.30
	11	< LOD	0.23	0.25	0.42
	12	< LOD	0.36	0.39	0.40
	13	< LOD	0.34	0.44	0.25
	14	< LOD	0.33	0.55	0.57
2nd week avg		< LOD	***0.30*** ± 0.06	***0.54*** ± 0.21	***0.39*** ± 0.11
3rd week	15	< LOD	0.28	0.44	0.38
	16	< LOD	0.49	0.40	0.35
	17	< LOD	0.52	0.30	0.48
	18	< LOD	0.61	0.24	0.38
	19	< LOD	0.44	0.22	0.23
	20	< LOD	0.24	0.39	0.19
	21	< LOD	0.47	0.63	0.34
3rd week avg		< LOD	***0.44*** ± 0.13	***0.37*** ± 0.14	***0.32*** ± 0.10
4th week reacclim	22	< LOD	0.42	0.68	0.47
	23	< LOD	0.33	0.21	0.17
	24	< LOD	0.30	0.11	0.08
	25	< LOD	0.08	<LOD	< LOQ
	26	< LOD	0.05	< LOQ	< LOQ
	27	< LOD	0.05	<LOD	<LOD
	28	< LOD	0.05	<LOD	<LOD
	29	< LOD	<LOD	<LOD	<LOD
4th week avg		< LOD	***0.18*** ± 0.16	***0.14*** ± 0.30	***0.10*** ± 0.18
mean[b]		0	**0.35** ± 0.14	**0.41** ± 0.19	**0.33** ± 0.11

[a] Determination limit of analytical method, LOD 0.01 μg DON kg^{-1}; limit of quantification, LOQ 0.03 μg DON kg^{-1}. [a] Values are mean ± std. dev. for samples containing detectable level of DON from 1 to 3 weeks

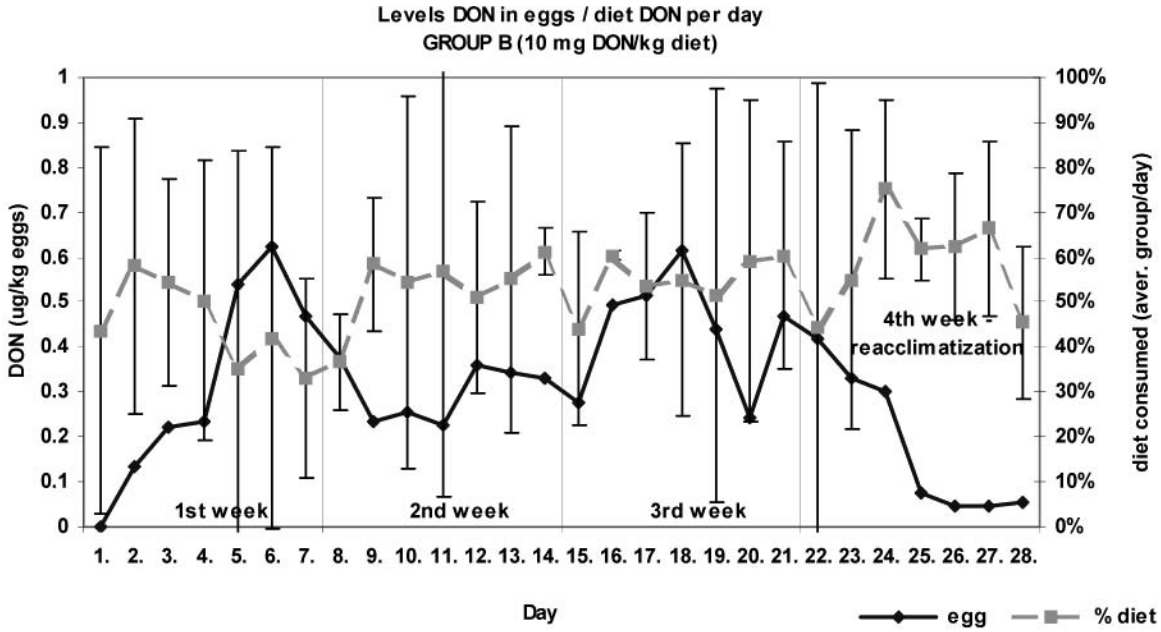


Figure 3. Levels of DON (μg kg^{-1}) found in eggs together with diet consumption (in % ± RSD) for the highest treatment level 10 mg DON kg^{-1} diet.

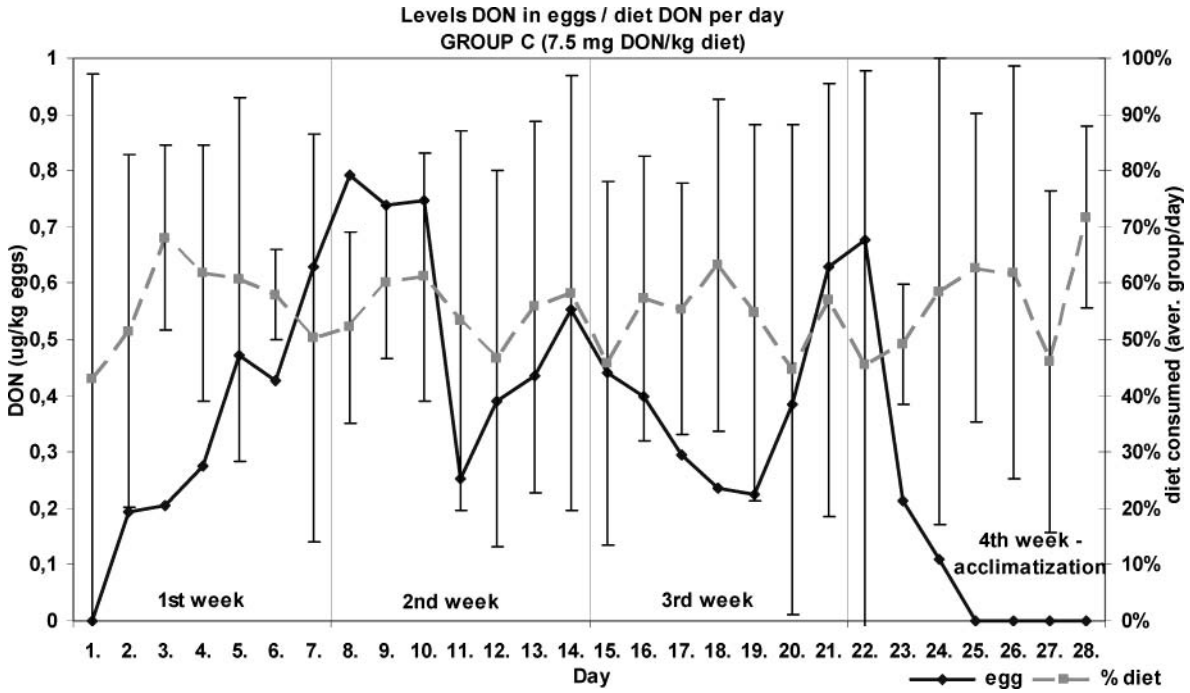


Figure 4. Levels of DON (μg kg^{-1}) found in eggs together with diet consumption (in % ± RSD) for the middle treatment level 7.5 mg DON kg^{-1} diet.

target analytes using eggs spiked at 1 μg kg^{-1} in each sequence of the analyzed samples (at the beginning and the end of the sequence). The average recoveries (± SD) obtained were 79.5 ± 15.2%, 74.0 ± 16.8%, 77.1 ± 15.0%, 78.6 ± 20.3%, 74.5 ± 14.4%, and 75.2 ± 18.3% for DON, ZON, α-ZOL, β-ZOL, α-ZAL, and β-ZAL ($n = 10$), respectively. The results can be considered satisfactory, considering the complex matrixes, complicated sample preparation, and analysis involved and the concentration of analyte determined. For quantification of compounds, an external standard procedure was used: uncontaminated eggs processed by the same procedure as the samples were subsequently spiked with toxin standards to achieve concentrations between 0.025 and 5 μg DON kg^{-1} sample and 0.3−5 μg kg^{-1} sample for ZON and its metabolites. Calibration graphs were accepted if the correlation coefficients were greater than or equal to 0.995. Retention time drift criteria were set at no more than ± 5% from the first to last standard injection of the LC run. Peak identification was confirmed by identifying the peak in the test extract and comparing the retention time with that of the nearest working standard injected in the LC run. A peak in a sample extract was discounted if its retention time differed by more than 2.5% from the standard peak. For each analyte, two highly abundant product ions were monitored (SIM mode), see **Table 2**. Confirmation of identity was made by comparing the ratios of the responses (peak areas or heights) for the different ions measured in the sample, with the response ratios obtained from a reference solution of the analyte analyzed under the same conditions. The ratio of the response, or the measured concentration for one or more additional ions, to the response of the quantitation ion fell between 0.8 and 1.2 of the mean ratio observed for standards. The limits of detection for DON and ZON (and its metabolites), considered as the minimum

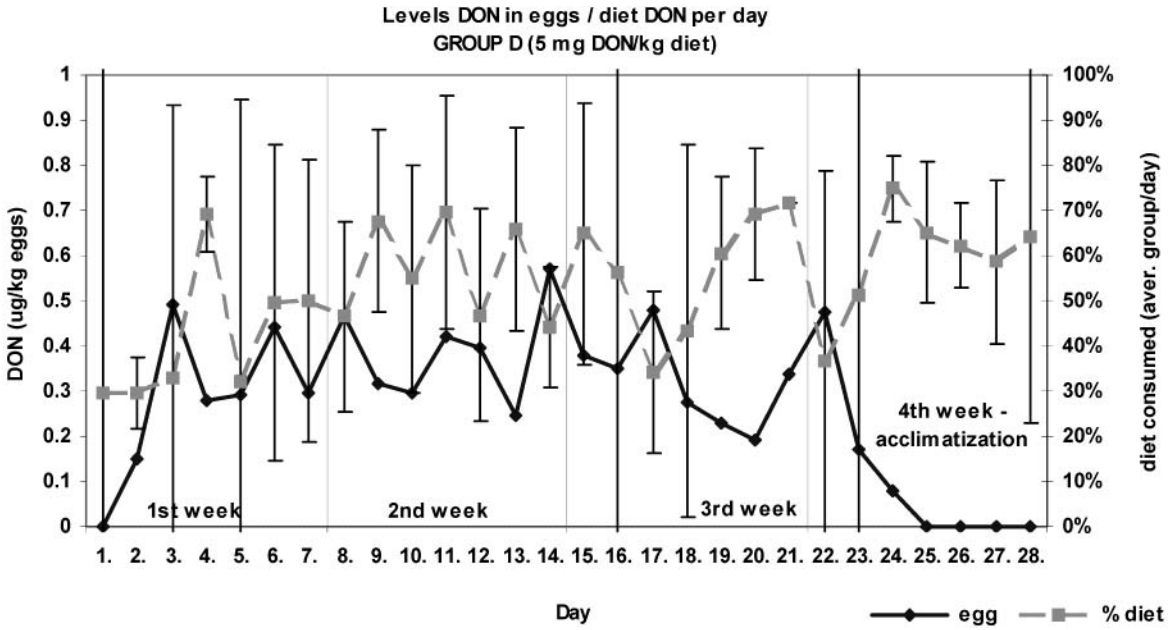


Figure 5. Levels of DON (μg kg^{-1}) found in eggs together with diet consumption (in % ± RSD) for the lowest treatment level 5 mg DON kg^{-1} diet.

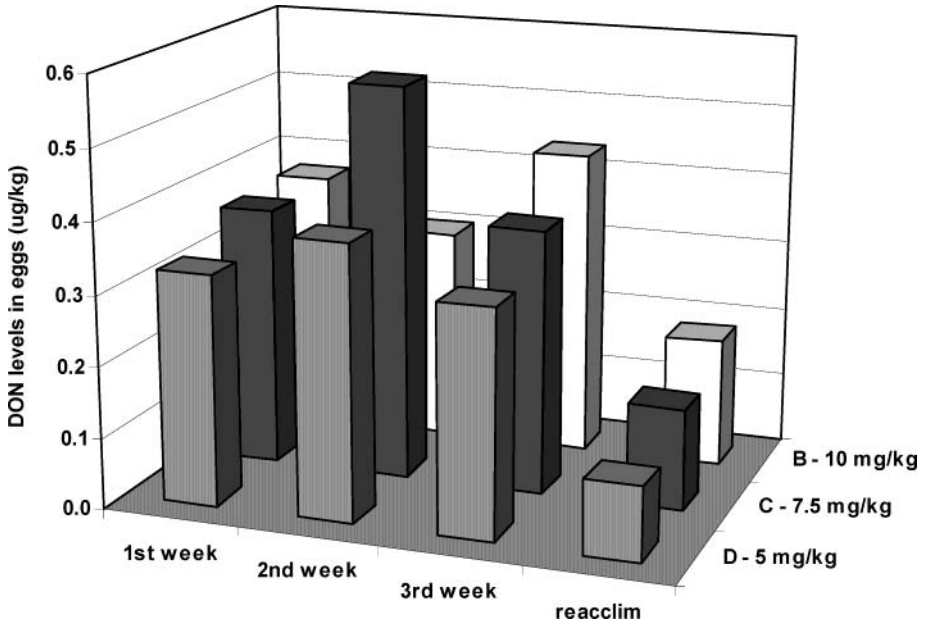


Figure 6. Means of DON levels in eggs for different treatment groups per week.

amount of toxin that could generate a chromatographic peak of three times greater than the baseline standard deviation, were 0.01 and 0.1 μg kg^{-1}, respectively. The concentrations of DON detected in eggs collected daily, pooled, and then homogenized for each treatment group are shown in **Table 5**. Residues of DON were detected in the pooled egg samples of all groups (B−D) receiving DON-contaminated rations. The levels found in samples during the 3 weeks of treatment with contaminated rations ranged from 0.13 to 0.79 μg kg^{-1} eggs, and with the mean levels of 0.33 ± 0.11, 0.41 ± 0.19, and 0.35 ± 0.14 μg kg^{-1} eggs for groups fed with 5, 7.5, and 10 mg DON kg^{-1} feed, respectively. Quantitative results were not corrected for recovery. No statistical difference between treatment groups for concentrations of DON in eggs was found; however, **Figure 6** suggests there may be a difference for weeks 1−3 and the re-acclimatization period, and it is discussed further in detail. A rapid rise of DON levels in the eggs was observed in the first 7 days of the feeding with contaminated rations (see **Figures 3**−**5**). After that period, for the next two weeks of treatment in all three groups, the levels of DON found in eggs oscillated around average levels of 0.35, 0.41, and 0.33 μg DON kg^{-1} eggs for treatment group 10, 7.5, and 5 mg DON kg^{-1} feed, respectively, presumably because of biological variation (the amount of diet consumed by each hen the day before), although all birds in the groups received an apparently identical dose. DON reached maximum levels of 0.61 (Day 18), 0.79 (Day 8), and 0.57 (Day 14) μg DON kg^{-1} eggs for treatment group 10, 7.5, and 5 mg DON kg^{-1} feed, respectively. DON levels in the case of the two lower treatment levels (group C, 7.5 mg DON kg^{-1} feed and group D, 5 mg DON kg^{-1} feed) were reduced to undetectable levels within 3 days of withdrawal of contaminated wheat from the hens' rations. For the highest treatment level (group B, 10 mg DON kg^{-1} feed) levels of DON were reduced to undetectable levels within 7 days of withdrawal of contaminated grain from the hens' diet. Mean levels of DON found in each group per week are shown in **Figure 6**. The reduction in

levels of DON from maximum values observed around week one of the study may be as a result of the hens' metabolism becoming more adept at excreting the toxin via other routes (e.g., urine, feces) in form of other compounds (i.e., de-epoxy, hydrolysis, and/or conjugate metabolites) (*25*, *26*). Parts of body organs (breast, kidney, liver, and ovary) susceptible to mycotoxins contamination were collected at the end of study to be available for any future work.

From the above data the transmission level of free DON was shown to vary between 0.002 and 0.004%, which is similar to the results reported by Lake et al. (*27*) from experiments carried out with a single dose of ^{14}C labeled DON administered to rats. The proportion of the dose found in most tissues of the rats was low, with only the liver and gastro-intestinal tract retaining more than 0.005% of the administered dose. Prelusky et al (*7*) reported DON/DON metabolites transmission level 0.31% to eggs according to the measurement of radioactivity. This higher transmission figure includes bound DON and metabolites, whereas using our analytical method, only free DON residues in eggs were determined. No digestion step was carried out during sample preparation to release conjugated material. The estimations of average feed to chickens and levels of DON found in eggs give transmission rate for residues of nonmetabolized DON after 21 days of exposure to contaminated diets at levels 5, 7.5, and 10 mg DON kg^{-1} feed of approximately 15 000:1, 18 000:1, and 29 000:1, respectively. These levels are similar to those mentioned by Trucksess et al. (*15*) for aflatoxin B_1 (40 000:1), and the main route of excretion of DON residues will probably be through the hens' body (urine) (*28*, *29*). No other data about transmission rate were found in the literature for DON or ZON.

All of the samples analyzed were negative for the presence of ZON or its metabolites (below LOD 0.1 μg kg^{-1}). This study shows, based on the level of ZON found in the contaminated wheat fed to the chickens and the LOD of analytical method used, that the transmission of ZON and its metabolites was below 0.03%.

There are no reports concerning *Fusarium* mycotoxins residues in eggs that discuss the distribution of residues between albumen and egg yolk. It is speculated that due to the lipophilic nature prolonged exposure to ZON could lead to an accumulation of the toxin in the egg yolk. Although we have not yet undertaken this work, the samples have been collected in such way as to facilitate this study in the future. Eggs containing naturally incurred mycotoxin residues will also facilitate future work on stability during storage and cooking. For eggs that are stored at temperatures above +4 °C the enzymatic system in the egg is still active, water and carbon dioxide are lost, and albumen physiochemical properties such as pH are changed. Eggs held under adverse environmental conditions can encourage mould growth (*24*). All these factors can affect the levels of mycotoxins or their metabolites, which could be found in eggs and could cause unexpected problems in sample preparation or analysis.

Conclusion. This paper reports the transmission of DON from naturally contaminated grain incorporated into chicken feed into eggs at levels corresponding to 0.002−0.004%. Although maximum levels of DON contamination of 10 mg kg^{-1} were relatively high, no adverse effects were observed on the laying hens, nor were there any adverse effects on egg production in terms of yield, weight of eggs, or shell thickness. The transmission of DON to eggs can lead to human dietary exposure, but this is probably not significant in view of the relatively low consumption of eggs on dietary weight basis, compared with other sources of exposure such as cereals and cereal based products. Levels of ZON in the hen feed at 0.5 mg kg^{-1} were too low for ZON or metabolites to be detected in eggs.

From the point of view of effects of consumption of the contaminated wheat in the hens diet (5−10 mg. kg^{-1}) it can be concluded that the *Fusarium* mycotoxin levels encountered had minimal effect on egg production.

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