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The effect of individual and mixtures of mycotoxins and persistent organochloride pesticides on oestrogen receptor transcriptional activation using *in vitro* reporter gene assays

Ukpai A. Eze ^{a, b, ψ}, John D. Huntriss^c, Michael N. Routledge^d, Yun Yun Gong ^{a, e*} and Lisa Connolly^f

^aSchool of Food Science and Nutrition, Food Science Building, University of Leeds, LS2 9JT, UK

^bDepartment of Medical Laboratory Sciences, Faculty of Health Sciences, Ebonyi State University, P. M. B. 053, Abakaliki, Nigeria

^cDivision of Reproduction and Early Development, Leeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, University of Leeds, LS2 9JT, UK

^dLeeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, University of Leeds, LS2 9JT, UK

^eDepartment of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, Ministry of Health, Beijing, 100021 PR China

^fInstitute for Global Food Security, School of Biological Sciences, Queens University Belfast, Northern Ireland, BT9 5AF, UK

***Corresponding Author (reprints):**

Yun Yun Gong PhD

School of Food Science and Nutrition, Food Science Building,
University of Leeds, Leeds, LS2 9JT, United Kingdom.

Tel: +44 (0) 1133431415; **E-mail:** y.gong@leeds.ac.uk

^ψ**Current address:** School of Life Sciences, Faculty of Health and Life Sciences, Coventry University, CV1 2DS, United Kingdom (**E-mail:** ad2179@coventry.ac.uk)

Abstract

The mycotoxins zearalenone (ZEN) and alpha-zearalenone (α -ZOL), which are common contaminants of agri-food products, are known for their oestrogenic potential. In addition to mycotoxins, food may also contain pesticides with oestrogenic properties such as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (*p,p'*-DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDE), raising the question on the potential effects of individual and combinations of these xeno-oestrogens on the action of natural oestrogens. The present study employed a mammalian reporter gene assay to assess the effects individual and binary combinations of these environmental and food-borne contaminants on oestrogen nuclear receptor (ER) transactivation. As expected, α -ZOL and ZEN exhibited the strongest oestrogenic potency (EC_{50} : 0.27 ± 0.121 nM and 1.32 ± 0.0956 nM, respectively) whereas *p,p'*-DDT and *p,p'*-DDE had weak ER agonistic activity with the maximal response of $28.70 \pm 2.97\%$ and $18.65 \pm 1.77\%$, respectively. Concurrent treatment of the mycotoxins and/or pesticides, individually or in binary combination, with 17β -oestradiol (E_2) showed either additive, synergistic or antagonistic interactive effects on E_2 -mediated ER response, depending on the combination ratios, the concentration range of xeno-oestrogens, and the concentration of E_2 . This study highlights the importance of assessing the mixture effects of chemical contaminants in risk assessment, especially in the area of reproductive and developmental toxicity.

Keywords: Mycotoxins; Chemical mixtures; Food-borne chemical contaminants; Reporter gene assay; Endocrine disruptor; Reproductive and developmental toxicity

1. Introduction

Oestrogen (E₂) plays an important role in cell growth, differentiation, and the proper functioning of both the male and female reproductive systems (Kuiper et al., 1998). This critical biological function of oestrogen is mediated through the oestrogen receptors (ER); ESR1 (ER α) and ESR2 (ER β), which are members of the nuclear receptor superfamily. There has been increasing evidence that many natural chemicals in food and synthetic environmental chemicals have the ability to interfere with the ER activity in both humans and animals resulting in endocrine disruption (Connolly, 2009). A few natural toxins in food (e.g. mycotoxins) and some synthetic chemicals (e.g. pesticides) are known to possess oestrogenic and/or anti-oestrogenic properties, and cause adverse reproductive health outcome in humans and animals, including precocious puberty and early thelarche in females, poor sperm quality, modified sexual behaviour, and alteration of the functions of reproductive organs in males and females (Diamanti-Kandarakis et al., 2009; Massart *et al.*, 2008, 2010; Connolly et al., 2011; Bittner et al., 2014; Kowalska et al., 2016). Zearalenone (ZEN) and its metabolites alpha-zearalenol (α -ZOL) and beta-zearalenol (β -ZOL) also exhibited endocrine disrupting effects at the level of nuclear receptor signalling and steroidogenesis using *in vitro* bioassays (Frizzell et al., 2011). There is the possibility of ZEN, α -ZOL and β -ZOL occurring in mixtures in biological systems as they are produced simultaneously by *Fusarium species* in corn stems (Minervini and Dell'Aquila, 2008) and can be found in edible tissues of farm animals (Dänicke and Winkler, 2015). Furthermore, as pesticides especially *p,p'*-DDT and *p,p'*-DDE are common contaminants of the environment and agricultural produce, co-occurrence with mycotoxins is inevitable (Romero-González et al., 2011; Akoto et al., 2013).

Research into the effects of mycotoxins, including ZEN, α -ZOL and β -ZOL on ER transcriptional activity has traditionally focused on the effects of single toxins (Kuiper et al., 1998; Shier et al., 2001; Frizzell et al., 2011; Cozzini et al., 2012; Molina-Molina et al., 2014; Ehrlich et al., 2015; Drzymala et al., 2015) while only a few studies have assessed the effects mixtures of these toxins may have on ER transcriptional activity (Demaegdt et al., 2016; Vejdovszky et al., 2017a, 2017b). However, multiple mycotoxin exposure is the rule and not the exception. For instance, the examination of 7049 feed and feedstuff from the Americas, Europe and Asia collected between 2009 and 2011 found that 48% had two or more mycotoxins, including aflatoxin B₁ (AFB₁), deoxynivalenol (DON), ZEN and ochratoxin A (OTA) (Rodrigues and Naehrer, 2012). In a subsequent study, feed, millet, maize and infant foods collected from Burkina Faso and Mozambique contained multi-mycotoxins (Warth et al., 2012). In addition, human exposure to multiple mycotoxins have been reported in several epidemiological studies using multi-mycotoxin biomarker method (Shephard et al., 2013; Warth et al. 2013). Multi-mycotoxin exposure poses a global public health threat as animal and *in vitro* studies have shown that additive, synergistic or antagonistic effects occur in mycotoxin mixtures (Alassane-Kpembi et al., 2013; Greiner and Oswald, 2011), highlighting the necessity of evaluating the health effects of mycotoxin co-occurrence.

It is well established that ZEN, α -ZOL and β -ZOL activate ER α and ER β both *in vitro* and *in vivo*, with α -ZOL showing the highest oestrogenic potency (EFSA, 2016, 2017; Tatay et al., 2018). The estrogenic potential of dichlorodiphenyltrichloroethane (DDT) and its isomers have also been investigated with 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDE) showing low agonistic activities on ERs whereas *o, p'*-DDT (1,1,1-trichloro-2(p-chlorophenyl)-2-(*o*-chlorophenyl) ethane) had strong oestrogenic activity (Chen et al., 1997; Brennan et al., 2016). However, there are no reports on

the effect of binary combinations of ZEN, α -ZOL and either other mycotoxins or DDT on E₂-mediated ER transcriptional activation. Therefore, the present study evaluated the effect of individual and mixtures of mycotoxins and/or persistent organochloride pesticides on ER nuclear transcriptional response in the absence or presence of E₂ (the endogenous ER agonist which will naturally vary in concentration) using the MMV-Luc reporter gene assay (MMV-Luc RGA).

2. Materials and methods

2.1 Mycotoxins and chemicals

Zearalenone (ZEN; purity $\geq$ 98%), alpha-zearalenol (α -ZOL; purity $\geq$ 98%), Deoxynivalenol (DON; purity $\geq$ 98%), ochratoxin A (OTA; purity $\geq$ 98%), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT; purity $\geq$ 98%), 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE; purity $\geq$ 98%), methanol (99.99%), 17 β -estradiol (E₂), fulvestrant (ICI 182,780; purity $\geq$ 98%), progesterone (P₄; purity $\geq$ 99%), sterile Dulbecco's phosphate buffered saline (PBS) and charcoal-stripped foetal bovine serum were purchased from Sigma-Aldrich (Dorset, England). The cell proliferation kit I (Cat. No: 11465007001) containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide labeling reagent (MTT; 5mg/mL in PBS) and solubilisation solution (10% Sodium dodecyl sulphate in 0.01 M hydrochloric acid) were obtained from Roche Diagnostics (Mannheim, Germany). Dulbecco's Modified Eagle Medium (DMEM) without phenol red, TrypLETM Express, CountessTM cell counting chamber slides and trypan blue were obtained from InvitrogenTM Life Technologies (Paisley, UK). All other chemicals used were standard laboratory grade. The stock concentrations of mycotoxins, hormones and fulvestrant were made in methanol and the final concentration of methanol in the culture medium was 0.5% (v/v) equivalent to the highest methanol concentration of

working dilutions. This was tested and results were not significantly different from media controls.

2.2 Reporter Gene Assay

The stably transfected MMV-Luc reporter gene cell line was a kind gift from Dr Marc Muller (University of Liège, Liège, Belgium) and was previously developed from the human mammary MCF-7 cell line (ATCC HTB-22) by transformation with a luciferase reporter construct under the control of an oestrogen inducible promoter, the MAR-Vit-Luc reporter plasmid (Willemsen et al., 2004). It is specific for the detection of oestrogen receptor transcriptional activation and endogenously expresses both alpha and beta oestrogen receptor, but predominantly alpha. The oestrogen-responsive element (ERE) construct vit-gpt was a gift from Gerhart U. Ryffel (Universitätsklinikum Essen, Essen, Germany) and previously cloned from *Xenopus* vitellogenin A2 (vit. A2) gene (Klein-Hitpass et al., 1988). Briefly, the pLuc reporter vector was developed through the insertion of a 2.0 Kb XhoI/StyI fragment from pXP2 containing the luciferase gene and the SV40 polyadenylation signal, into the promoterless pBLCAT6 vector opened by XhoI/StyI (Willemsen et al., 2004). Thereafter, the HindIII fragment of the *Xenopus* A2 vitellogenin promoter was isolated from Vit-gpt vector (Klein-Hitpass et al., 1988) and inserted into the HindIII-opened pLuc vector. This resulted in the generation of the Vit-Luc firefly luciferase reporter construct. Furthermore, a HindIII/EcoRI (800 bp) fragment containing a nuclear scaffold-attached region sequence was inserted into the StuI site upstream of the Vit promoter to generate a stably integrated MAR-Vit-Luc vector (Willemsen et al., 2004). Then, a MCF-7 human breast cancer cell line (ATCC HTB-22) which expresses both ER- α and ER- β receptors (Al-Bader et al., 2011) was transformed with the MAR-Vit-Luc reporter plasmid to obtain the MMV-Luc cell clone (Willemsen et al., 2004).

The oestrogenic response obtained from the mammalian-based ER reporter gene cell lines has been shown to be well correlated with the response observed using the gold standard for oestrogenic assay '*in vivo* uterotrophic assay in immature or ovariectomised rodents' (Wang et al., 2014a,b), providing faster, easier and cheaper alternative testing strategy for oestrogenicity.

2.2.1 Cell culture

MMV-Luc cells were cultured in DMEM (without phenol red) containing 10% charcoal-stripped foetal bovine serum without antibiotics and maintained in a 75 cm² cell culture flask (Nunc, Roskilde, Denmark) at 37 °C in a humidified atmosphere (95%) with 5% CO₂ for at least 48 h before use to ensure that the cells are completely free from hormonal induction from the media. For experiments, attached cells were liberated from flasks using TrypLE™ Express trypsin (Invitrogen™ Life Technologies, Paisley, UK), counted for viability checks prior to seeding plates by trypan blue staining and using a Countess® automated cell counter (Invitrogen™ Life Technologies, Paisley, UK). Cells (100 µL) were seeded at a density of 4 x 10⁵ cells/mL into each well of white walled 96-well plates with clear flat bottoms (Greiner Bio-One, Frickenhausen, Germany) and incubated for 24 hours to allow cells to attach before chemical treatment.

2.2.2 Effects of individual mycotoxins and pesticides on ER transcriptional response

The cells (4 x 10⁵ cells/mL per well) were exposed to different concentrations of mycotoxins, persistent organochloride pesticides, reference hormones and fulvestrant (ICI 182,780; ER antagonist) diluted with media to the final concentrations: ZEN (0.01 – 1000 nM), α -ZOL (0.005 – 50 nM), DON (1 – 30,000 nM), OTA (1 – 30,000 nM), *p,p'*-DDT (1.0 – 50.0 µM), *p,p'*-DDE (1.0 – 50.0 µM), E₂ (0.0005 – 10 nM), fulvestrant (ICI 182,780; 1 µM), progesterone

(500 nM), and methanol (0.5%) and media controls. The MMV-Luc reporter gene assay was performed as previously reported (Frizzell et al., 2011; Ndossi et al., 2012), except that cells were treated with chemicals diluted with media without antibiotics and were incubated for 48 h instead of 24 h. The presence of antibiotics in media causes high background luciferase response (Wilson et al., 2004). After incubation for 48 hours, the media supernatants were discarded, the cells washed once with sterile PBS and lysed with 25 μ L of lysis buffer (Promega, Southampton, UK; Cat. No.: E1531). Finally, 100 μ L of luciferase enzyme (Promega, Southampton, UK; Cat. No.: E1501) was injected into each well and the luciferase activity in relative light units (RLUs) were measured using the Mithras LB 940 Multimode luminometer (Berthold, Other, Germany). The luminescence readings of each treatment was normalised to the readings of E₂ alone (10 nM) and this was taken as the maximum response (100%). Vehicle (0.5% methanol) controls were used to define the minimum response (0%). This gave the relative response of the cell line to each compound was calculated and compared with activity of 0.5% methanol and E₂ (10 nM) arbitrarily set at 0% and 100%, respectively. Measurements of 10 nM E₂, 0.5% methanol and progesterone were conducted in parallel on every single plate and served as positive, solvent and negative controls, respectively.

2.2.3 Effects of individual and mixtures of mycotoxins and pesticides on E₂-mediated ER response

The mycotoxins (ZEN and α -ZOL) and pesticides (*p,p'*-DDT and *p,p'*-DDE) with relative estrogenic response above 10% were selected and tested together with E₂ (0.05 nM and 10 nM) either in single or binary combinations to ascertain their additive and antagonistic effects on E₂-mediated ER transcriptional response. The 0.05 nM and 10 nM of E₂ were chosen as they fall within the reference ranges of serum concentrations of E₂ seen in females in pre-

pubertal stage, during puberty and in late pregnancy (JECFA; 2000; Elmlinger et al., 2002). The normal serum E₂ concentration is usually in the range 8 – 18 pg/mL (0.029 – 0.066 nM) in prepubertal women, 20 – 350 pg/mL (0.073 – 0.35 nM) in premenopausal women and this reaches peak level (18,000 pg/mL: 66.1 nM) in late pregnancy (JECFA; 2000; Elmlinger et al., 2002). Furthermore, circulating E₂ level ranges $\leq$ 10 – 50 pg/mL (0.037 – 0.184 nM) both in prepubertal and adult men, depending on age (JECFA; 2000). Serum concentration of ZEN (0.628 - 1.492 μ M), α -ZOL (0.218 - 2.207 μ M) and β -ZOL (0.343 - 2.207 μ M) have been reported in individuals with breast and cervical cancer (Pillay et al., 2002), and this is similar to those used in this study. In addition, serum *p,p'*-DDT and *p,p'*-DDE concentrations of 40.311 μ M and 86.155 μ M, respectively have been reported in human exposure studies (Dua et al., 2001; Longnecker et al., 2002).

The concentrations of ZEN (0.01 nM - 1000 nM), α -ZOL (0.005 nM - 50 nM), *p,p'*-DDT (1 μ M - 50 μ M) and *p,p'*-DDE (1 μ M - 50 μ M) were used in single or binary combinations tested together with the selected concentrations of E₂. After exposure of the cells to the mycotoxins and pesticides together with E₂ (0.05 nM and 10 nM) either in single or binary combinations for 48 hours, the media supernatants were discarded, the cells washed once with PBS and lysed with 25 μ L of lysis buffer (Promega, Southampton, UK). Finally, 100 μ L of luciferase enzyme (Promega, Southampton, UK) was injected into each well and the luciferase activity in relative light units (RLU's) were measured using the Mithras Multimode Reader (Berthold, Other, Germany). The relative response of the cell line to each compound was calculated and compared with activity of 0.5% methanol and E₂ (0.05 nM and 10 nM) arbitrarily set at 100%. Measurements of E₂ (0.05 nM and 10 nM), 0.5% methanol and progesterone were conducted in parallel on every single plate and served as positive, solvent and negative controls, respectively. The high affinity ER antagonist ICI 182,780 was used to verify whether the

detected estrogenic responses are ER dependent. E₂ (0.05 nM and 10 nM) was co-incubated with 1 µM of ICI 182,780 and the inhibition of oestrogenic response of E₂ confirmed that the responses observed in the assay were mediated oestrogen receptors (ERs). Progesterone was tested in each plate as a negative control and it had no estrogenic response.

2.3 Cell viability and cytotoxicity assay

The MMV-Luc cells were seeded and treated as described for reporter gene assay (RGA). After 48 hours of treatment, 20 µL of MTT labeling reagent (5mg/mL) was added into each well containing cells growing in 200 µL of medium and plates covered with aluminum foil. The plates were incubated for 4 hours at 37 °C in a humidified atmosphere (95%) with 5% CO₂. Thereafter, 100 µL of solubilisation solution (10% SDS in 0.01 M HCl) was added to each well and incubated overnight at 37 °C in a humidified atmosphere (95%) with 5% CO₂. The plates were then read at 570 nm with a reference wavelength of 690 nm with TECAN Safire2 (TECAN, Switzerland) microtitre plate reader. Viability of the each test sample was calculated as the percentage (%) absorbance when compared with the absorbance of the 0.5% methanol vehicle control.

2.4 Statistical analysis

Both RGA and MTT assay exposures were carried out in triplicate wells and in three independent experiments. Results were expressed as the mean ± standard deviation (Mean ± SD) of the triplicate exposures. For the RGAs, relative dose response (fold induction) was calculated from the relative light units (RLUs) when compared with the negative control (*n*-fold) using Microsoft Excel (Microsoft, Redmond, Washington) and normalised with the response of E₂ (10 nM) arbitrarily set at 100%. The dose-response curves were fitted with

GraphPad PRISM software version 5.0 (San Diego, CA) using the sigmoidal dose-response curve equation, $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{EC}_{50} - X)})$, where X is the logarithm of concentration, Y (the response), and Bottom and Top are set at 0% and 100% for the lowest and maximal transcriptional response of the assay. A one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was used to determine significant differences between the treatments and the corresponding controls in the RGAs and MTT assays. The mean concentrations were tested for significant difference at the 95% confidence level. A p value of < 0.05 was considered statistically significant, $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***) and ≤ 0.0001 (****). The half maximal effective concentration (EC_{50}) for the single compounds were determined by fitting dose-response curves using GraphPad PRISM version 6.0.

The interactive effects of the combined toxins on E_2 -mediated ER transcriptional response were calculated as previously described by Weber et al. (2005) and Clarke et al. (2014) and briefly shown below.

Mean ER response of binary mixtures (expected in % of substance 1 + substance 2)

= mean ER response (substance 1 in %) + mean ER response (substance 2 in %) -
maximal ER response by 10 nM of E_2 (100%).

The standard deviation (SD) was calculated using the model described by Weber et al. (2005) as shown below:

$$\text{Expected SD (substance 1 + substance 2)} = [(\text{SD of substance 1})^2 + (\text{SD of substance 2})^2]^{1/2}$$

In order to evaluate the interactive effects of the combined toxins below or above additivity, expected additive values were compared to actually measured values using a multiple t-test and corrected for multiple comparison using Holm-Šidák test method, with $p < 0.05$.

The results were interpreted as follows:

- **Additive effects:** measured ER response values were not significantly above or below the expected values.

- **Synergistic effects:** measured ER response values were significantly below expected values.
- **Antagonistic effects:** measured ER response values were significantly above expected values.

3. Results

3.1 Cell viability and cytotoxicity

The single mycotoxins, pesticides and E₂ did not reduce the cell viability of MMV-Luc cell line after 48 h in all the concentrations tested (**Supplementary file 1**). It was found that ZEN and E₂ significantly increased cell proliferation ($p \leq 0.001$) whereas α -ZOL slightly increased cell proliferation at 1 μ M and 50 μ M ($p \leq 0.05$). In the concurrent exposure of either single or binary mixtures of mycotoxins and pesticides with E₂ at 0.05 nM and 10 nM, no cytotoxic effect was observed (**Supplementary file 1**) indicating that the transcriptional responses observed are not as a result of reduced cell viability.

3.2 ER transcriptional response induced by single mycotoxins and persistent organochloride pesticides

The maximum induction of the E₂ (10 nM) standard in MMV-Luc reporter gene assay (RGA) was set at 100% in determining the transcriptional response of the other environmental and food-borne contaminants. Therefore, the response from other compounds is relative to this maximal response by 10 nM of E₂. The dose-response curve for the ER transcriptional activation of the single mycotoxins and pesticides is shown in **Figure 1**. Among the single compounds tested, E₂ at 1.0 nM activated ER transcriptional response closely to the maximum induction ($95.93 \pm 3.92\%$) (**Table 1.1**). The highest transcriptional activation for ZEN was at

1 μ M ($80.11 \pm 5.58\%$) whereas its major metabolite α -ZOL at 50 nM induced similar response ($73.45 \pm 3.25\%$) (**Table 1.1**). A dose response curve was plotted (**Table 1.1**), E₂ had an EC₅₀ of 0.053 ± 0.012 nM while ZEN and α -ZOL had an EC₅₀ of 1.32 ± 0.10 nM and 0.27 ± 0.12 nM, respectively. In this study, *p,p'*-DDT and *p,p'*-DDE also weakly activated ER transcription with the highest response occurring at 50 μ M, $28.70 \pm 2.97\%$ and $18.65 \pm 1.77\%$, respectively (**Table 1.1**).

3.3 Effects of single mycotoxins and persistent organochloride pesticides on E₂-mediated ER transcriptional response

It was observed that co-incubation of either ZEN or α -ZOL with E₂ (0.05 nM) significantly ($p \leq 0.001$) modulated the ER transcriptional response induced by E₂, alone (**Fig. 2A & 2C**) with more induction occurring at lower doses (0.01 μ M - 2.5 μ M) in each case. On the contrary, co-incubation of the same doses of either ZEN or α -ZOL with 10nM of E₂ caused a dose-dependent inhibition of ER response (**Fig. 2B & 2D**) reducing E₂-mediated response by approximately 40% at 10 μ M, in each case. Concurrent treatment of MMV-Luc cells with *p,p'*-DDT or *p,p'*-DDE with E₂ (0.05 nM or 10 nM) significantly reduced the ER transcription activation caused by E₂, alone (**Fig. 2E – 2H**).

3.4 Effects of binary mixtures of mycotoxins and persistent organochloride pesticides on E₂-mediated ER transcriptional response

Co-treatment of ZEN + α -ZOL, ZEN + *p,p'*-DDT, or ZEN + *p,p'*-DDE with E₂ (0.05 nM) significantly ($p \leq 0.001$) enhanced the transcriptional response induced by E₂ alone at lower concentrations whereas little or no effects were observed at higher concentrations (**Fig. 3A, 3C & 3E; Supplementary Fig. S2.1**). In contrast, co-exposure of E₂ (10 nM) with ZEN + α -ZOL, ZEN + *p,p'*-DDT, or ZEN + *p,p'*-DDE significantly decreased the E₂-mediated ER response

dose-dependently ($p \leq 0.001$), with the highest doses combinations reducing ER transcriptional response to approximately 40% (**Fig. 3B, 3D & 3F; Supplementary Fig. S2.1**).

This study also tested the ER transcriptional activation that will be elicited by concurrent exposure of E₂ (0.05 nM or 10 nM) with different dose combinations of α -ZOL + *p,p'*-DDT, α -ZOL + *p,p'*-DDE or *p,p'*-DDT + *p,p'*-DDE (**Fig. 4**). It was noted that α -ZOL + *p,p'*-DDT and α -ZOL + *p,p'*-DDE significantly ($p \leq 0.001$) modulated the transcriptional response of E₂ at 0.05 nM, but strongly inhibited the ER response mediated by E₂ (10 nM) with the highest dose combinations (α -ZOL: 10 μ M; *p,p'*-DDT: 50 μ M and *p,p'*-DDE: 50 μ M) reducing E₂-mediated ER transactivation response by over 70% (**Fig. 4A – 4D; Supplementary Fig. S2.2**). Co-treatment of equimolar mixtures of *p,p'*-DDT and *p,p'*-DDE with E₂ at 0.05 nM or 10 nM caused a dose-dependent decrease in E₂-mediated ER transactivation response and this was completely inhibited at equimolar concentrations (40 μ M and 50 μ M) for 0.05 nM of E₂ co-treatment and at 50 μ M for 10 nM of E₂ (**Fig. 4E & 4F; Supplementary Fig. S2.2**).

4. Discussion

There has been an increasing concern that environmental and dietary chemicals cause endocrine disruption leading to potential adverse effects on both animal and human reproduction (Diamanti-Kandarakis et al., 2009). In this study, the MMV-Luc cell line expressing both ER- α and ER- β receptors was employed to determine the effects of single and mixtures of mycotoxins and/or persistent organochloride pesticides on oestrogen receptor transcriptional activity. Since human reproductive organs such as testis, prostate, ovary, pre-menopausal and foetal uteri, and endometrium contain significant amounts of both ER- α and ER- β receptors (Mosselman et al. 1996; Brandenberger et al., 1997, 1998, 1999), and given the

critical role that ER- β plays in modulating the functional activity and levels of ER- α , the use of reporter cell lines with one ER subtype (ER- α or ER- β) may underestimate the overall estrogenic potency of a sample (Brennan et al., 2016). This is true as oestrogenic chemicals that were not detected using BG1Luc4E2 cells expressing only ER- α were identified by the BG1LucER β c9 cells which constitutively expresses both ER- α and ER- β (Brennan et al., 2016). In addition, ER transcriptional response mediated by *p,p'*-DDT, heptachlor, lindane, endrin, tedion, thiodan, methoxychlor and *o,p'*-DDT were higher in reporter cells with both ER- α and ER- β compared to ER α -containing reporter cells, indicating the modulation of the expression of ER- α target genes by ER- β or ER- β -specific gene induction (Bardin et al., 2004) and therefore, the use of reporter cells expressing only ER- α or ER- β but not both may underestimate the overall potency of (anti-)oestrogenic compounds and/or mixtures (Brennan et al., 2016).

4.1 Transcriptional activation of ER by single mycotoxins and persistent organochloride pesticides

Among the single compounds tested, E₂ at 1.0 nM and 10.0 nM activated ER transcriptional response by approximately $95.93 \pm 3.92\%$ and 100% induction, respectively. ZEN induced approximately $80.11 \pm 5.58\%$ ER transcriptional response at 1 μ M whereas its major metabolite α -ZOL induced approximately $73.45 \pm 3.25\%$ ER transcriptional response at 50 nM, relative to the response exhibited by 10 nM E₂ (100%). This is in agreement with the result of Ehrlich et al. (2015) who reported that ZEN and its metabolites (0.05 pM – 0.5 μ M) induced maximum ER transcriptional response of 60 – 75% in human oestrogen receptor-chemically activated luciferase expression (hER-CALUX) assay. However, in human 293 embryonal kidney (HEK) cells expressing luciferase (Luc) and β -galactosidase (LacZ), the transcriptional

response of ZEN on ER α and ER β at 1 μ M was 91% and 27%, respectively (Kuiper et al., 1998). The EC₅₀ values of each compound were measured from a dose response curve and was used as a representative of oestrogenic potency. In this study, E₂ strongly induced oestrogenic response with an EC₅₀ value of 0.053 ± 0.012 nM which is similar to most previously reported EC₅₀ values of E₂ using different assay methods (Jobling et al., 1995; Balaguer et al., 1996; Sonneveld et al., 2005; Brennan et al., 2016). However, this EC₅₀ value of E₂ (0.053 ± 0.012 nM) was slightly higher than the ones reported by Frizzell et al. (2011) and Demaegdt et al. (2016) using the same oestrogenic responsive (MMV-Luc) cell line. It is possible incubation of chemicals in cells for longer duration (e.g. 48 h) compared to shorter duration (e.g. 24 h) could affect their oestrogenic potency possibly due to metabolic capacity of cells. The comparison of the EC₅₀ data of ZEN and its major metabolite α -ZOL was also in agreement with previous studies with α -ZOL showing an increased oestrogenic potency compared with the parent compound ZEN (Frizzell et al., 2011; Molina-Molina et al., 2014; Ehrlich et al., 2015; Drzymala et al., 2015; Demaegdt et al., 2016). Although the ranking of oestrogenicity of ZEN and α -ZOL was similar to report of previous studies, the EC₅₀ values (ZEN: 1.32 ± 0.0956 nM and α -ZOL: 0.27 ± 0.121 nM) varied from those reported by Frizzell et al. (2011) and Demaegdt et al. (2016) using the same reporter cell line. It has been shown that the use of antibiotics or antifungals in culture media contribute to high background levels of luciferase response in T47D-KBLuc reporter cell line (Wilson et al., 2004). This can also be corroborated by the work of Covaleda and co-workers which highlighted that the presence of tetracycline in assay media inhibited ER β protein expression and modulated E₂-induced cell proliferation compared to that observed in the absence of tetracycline (Sotoca Covaleda et al., 2008). One major drawback of using the ER reporter assay over the activation of endogenous ER gene targets is that the stability of the luciferase reporter gene transfected into the cells could affect their luciferase responsiveness (Wang et al., 2012). In addition, unstable transfection can lead

to a loss of the luciferase reporter gene construct from the genome of the cells resulting in variations in absolute luminescence values. Interestingly, a stable transfection of the luciferase reporter gene occurred in the MMV-Luc cell line used in this study (Willemsen et al., 2004).

The organochloride pesticide *p,p'*-DDT and major metabolite *p,p'*-DDE showed weak oestrogenic response with the highest concentrations tested (50 μ M) showing response of $28.70 \pm 2.97\%$ and $18.65 \pm 1.77\%$ relative to the maximal response induced by E_2 (100%), respectively. It was noted that *p,p'*-DDT significantly induced ER transcriptional response at 1 μ M and above whereas significant response was only observed at 25 μ M for *p,p'*-DDE. In a yeast-based reporter gene assay, *p,p'*-DDT and *p,p'*-DDE were ER agonists with *p,p'*-DDT significantly inducing the β -galactosidase activity at concentration of 1 μ M and a 20 % response (EC_{20}) found at 51 μ M (Li et al., 2008). In a more recent studies, *p,p'*-DDT (10 μ M) induced an ER response of $19.0 \pm 5\%$ in an $ER\alpha$ expressing reporter (BG1Luc4E2) cell line and $30 \pm 3\%$ in a reporter cell line (BG1LucERB β 9) expressing both $ER\alpha$ and $ER\beta$ (Brennan et al., 2016), similar to the results of this study. However, *p,p'*-DDE was a more potent agonist in a MELN reporter cell line with an EC_{50} of approximately 26 μ M (Pillon et al., 2005) whereas *p,p'*-DDT only showed weak agonistic activity (7%) in HEK cells expressing $ER\alpha$ with luciferase reporter (Kuiper et al., 1998). In this study, DON and OTA had no oestrogenic response, similar to other studies using the same reporter cell line (Ndossi et al., 2012; Frizzell et al., 2013).

Oestrogens are essential in many reproductive function and can influence the growth, differentiation, and function of many target tissues in the reproductive system, including uterus, vagina, ovary, testes, epididymis, and prostate (Kuiper et al., 1998). In humans, about 1-2% of

circulating E₂ is unbound while 40% is bound to sex hormone-binding globulin (SHBG) and the remaining fraction to albumin (JECFA; 2000). Currently, it is not clear whether ZEN and its metabolites interact or bind to carrier proteins (e.g. SHBG and albumin) as exhibited by endogenous hormones. In *in vitro* studies, the binding capacity of ZEN on SHBG from humans and cold-water fish Arctic charr (*Salvelinus alpinus*) was poor and therefore, the concentrations applied *in vitro* could have greater access to ER binding sites which may induce more response *in vivo* compared to endogenous oestrogens (Metzler et al., 2010; Molina-Molina et al., 2014). In addition, ZEN is known to activate the human pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) in HepG2 hepatoma cells, indicating that it can also mediate its effect through other nuclear receptors (Ayed et al., 2001). Also worthy of note, it has been speculated that ZEN and its metabolites, especially α -ZAL, α -ZOL and β -ZAL may act as androgen receptor (AR) antagonists as their half maximal inhibitory concentration (IC₅₀) values were within the same range as reported for two well-known androgen receptor antagonists vinclozolin and flutamide in various reporter gene assays (Molina-Molina et al., 2014; Stypula-Trebas et al., 2016). On the other hand, *p,p'*-DDE bioaccumulates in lipophilic tissues which are released into the blood and breast milk posing infant health risk as a result of its action as both ER and AR antagonists (Kelce et al., 1995, 1997). Therefore, it is possible that co-exposure of these mycotoxins and pesticides could lead to additive or synergistic effects on E₂-mediated ER activation.

4.2 Interactive effects of single mycotoxins and pesticides on E₂-mediated ER transcriptional activation

It was observed that co-treatment of ZEN or α -ZOL with 0.05 nM of E₂ significantly enhanced E₂-mediated ER response in MMV-Luc reporter cell line in all the doses tested. In a previous study using the same MMV-Luc reporter cell line, Wielogórska and co-workers (2015) showed

that butyl 4-hydroxybenzoate (PB–Bu) and propyl 4-hydroxybenzoate (PB–Pr) which exhibited strong oestrogenic response also enhanced E₂-mediated response when co-exposed with 0.005 nM of E₂ (PB–Bu: 584.0% and PB–Pr: 460.0%). Also in agreement with the result of this study, several combinations of strong oestrogenic compounds ZEN and alternariol (AOH) or α -ZOL and AOH, at several concentrations had synergistic interaction and enhanced Ishikawa cell alkaline phosphatase production which exceeded the maximum induction (100%) reached by 1 nM of E₂ (Vejdovszky et al., 2017a). This current study also observed that combination of ZEN (0.01 - 10 nM) and α -ZOL (0.01 - 10 nM) with the concentration of E₂ (10 nM), which showed maximal effect on ER transcriptional activation, resulted in significant dose-dependent reduction of E₂-mediated ER transcriptional response, indicating that combination of ZEN and α -ZOL act as partial agonists and competes with E₂ for ER. In a more recent study, Vejdovszky et al. (2017b) demonstrated that binary combinations of genistein with ZEN or AOH resulted in either synergism or antagonism in the alkaline phosphatase assay, depending on the combination ratios and the concentration range. They also posited that the nature of interactions between two strong oestrogenic compounds may depend on the concentration ratio of the substances in the mixture and also on the concentration range applied. Furthermore, Wielogórska et al. (2015) demonstrated that the ER agonist di-*n*-butyl phthalate (DBP) at concentration of 1 μ M had no significant effect the E₂-induced ER transcriptional activation when combined with E₂ at 0.1 nM, but exhibited antagonistic effect at 10 μ M and 100 μ M, reducing transactivation by 23% and 75%, respectively and this was not as a result of cytotoxic effect on cells.

Furthermore, ZEN has been shown to be a mixed agonist/antagonist of both ER- α and ER- β receptors (Kuiper et al., 1998; Mueller et al., 2004) whereas other phytoestrogens, including resveratrol, enterolactone, 6-hydroxy- enterolactone are mixed weak agonist/antagonist of the ER- α in diethylstilbestrol-induced, ER-mediated activity in Ishikawa-

hER α and Ishikawa-hER β cells (Mueller et al., 2004). In addition, ZEN acted as an agonist for both ER- α and ER- β receptors (an endocrine disruptor) at low doses, but had ER antagonistic activity at high doses (Mueller et al., 2004). Bowers et al. (2000) also reported that resveratrol had slight antagonistic properties on ER α but not ER β depending on the ERE sequence. Partial ER-agonists enterolactone, 6-hydroxy-enterolactone acted as partial ER- α antagonists, reducing diethylstilbestrol mediated-ER transactivation by 20–40% (Mueller et al., 2004). Similar to the result of our study, the xenoestrogen resveratrol increased the diethylstilbestrol-induced transactivation of ER α and ER β at low doses, but at high doses it inhibited activity of ER α and ER β (Mueller et al., 2004). Therefore, mixtures of either ZEN or α -ZOL with E₂ (0.05 nM) may be able to elicit a more potent oestrogenic or anti-oestrogenic response than initially anticipated, posing a greater risk to reproductive health.

This study also demonstrated that co-exposure of *p,p'*-DDT or *p,p'*-DDE (especially at ≥ 5 μ M) with either 0.05 nM or 10 nM of E₂ significantly reduced the E₂-mediated ER transcriptional activity, indicating antagonistic effects (**Fig. 4E & 4F; Supplementary Fig. S2.2**). In a yeast-based hER reporter gene assay, combination of low dose of E₂ (0.3 nM) with low concentrations (40 nM - 1 μ M) of *p,p'*-DDT, *o,p'*-DDT or *o,p'*-DDE caused dose-dependent increase in transcriptional response, but this was not significantly different from the sum of the separate responses observed in 0.3 nM of E₂ or DDT isomers, alone (Chen et al., 1997). In addition, co-treatment of the above DDT isomers at equimolar concentrations (40 nM and 80 nM) produced additive transcriptional response in a concentration-dependent manner (Chen et al., 1997). The authors concluded that co-exposure of DDT isomers and metabolites with E₂ or another DDT isomer and metabolite can have an additive effect on hER transcriptional activation.

4.3 Interactive effects of the mixtures of mycotoxins and pesticides on E₂-mediated ER transcriptional activation

Since human beings and animals are not exposed to food and environmental oestrogenic chemicals alone, but in mixtures with other oestrogenic chemicals, this study also examined the effect of such mixtures on E₂-induced hER transcriptional activity. It was observed that mixtures of ZEN and α -ZOL, ZEN and *p,p'*-DDT, ZEN and *p,p'*-DDE, α -ZOL and *p,p'*-DDT, and α -ZOL and *p,p'*-DDE when co-treated with E₂ (0.05 nM) enhanced E₂-induced hER transcription at low dose combinations, but had additive or antagonistic effects at high dose combinations. However, the above mixtures caused dose-dependent reduction (antagonistic effects) on E₂-induced hER transcription when co-exposed with 10 nM of E₂. It has also been demonstrated that co-treatment of low concentrations of oestrogen agonists 4-Nonylphenol, *o,p'*-DDT, methoxychlor, chlordane, endosulfan and dieldrin at 200 nM to 2 μ M with low dose of E₂ (0.003 nM) in T47D-ER CALUX assay caused induction of transcriptional response which were significantly different from the response of the individual treatments (Legler et al., 1999), similar to the result of this study. These results indicate that oestrogenic chemicals may act synergistically or antagonistically depending on combination ratio or concentration and further research is warranted in the in vivo effects of mixtures of xeno-oestrogens.

A major observation of this study is that exposure of equimolar concentrations of *p,p'*-DDT and *p,p'*-DDE mixtures with either 0.005 nM or 10 nM of E₂ significantly reduced transcriptional response in concentration-dependent fashion and this response was completely inhibited at equimolar combinations (40 and 50 μ M) for 0.05 nM of E₂ or 50 μ M exposure with 10 nM of E₂. This observation was not as a result of cell cytotoxicity as there was either a significant increase in cell number or no effect on cell viability at these concentrations

(**Supplementary file 1**). In agreement with this study, the β -galactosidase activity was completely abolished when *p,p'*-DDT (100 μ M) and *p,p'*-DDE (100 μ M) were combined with 0.2 nM of E₂ in a yeast-based hER reporter gene assay, but significantly increased progesterone-mediated PR transcriptional activation (Li et al., 2008). Interestingly, serum *p,p'*-DDT and *p,p'*-DDE concentrations of 40.311 μ M and 86.155 μ M, respectively have been reported in human epidemiological studies (Dua et al., 2001; Longnecker et al., 2002) indicating clinical relevance of the effects observed in this study.

In conclusion, this study shows that oestrogenic mycotoxins and persistent organochloride pesticides, individually or in combination, have biphasic effects on E₂-mediated ER transcriptional response depending on the concentration of E₂ present as well as the concentration of each compound during co-exposure. Modulatory interaction effects were observed for low concentrations of ZEN + α -ZOL, ZEN + *p,p'*-DDT, and α -ZOL + *p,p'*-DDT mixtures when combined with physiologically relevant concentrations of E₂ whereas antagonistic effects were observed at high dose combinations. Of greatest concern is the finding that mixtures of equimolar concentrations of *p,p'*-DDT and *p,p'*-DDE antagonised the ER transcriptional response mediated by physiologically relevant concentrations of E₂ both in low and high concentrations, and that combinations at 50 μ M completely inhibited the E₂ mediated response. This study demonstrates the importance of assessing the interactive effects of oestrogenic compounds, individually or in mixtures, with physiologically relevant concentrations of E₂ during risk assessment. The mechanisms through which mycotoxins and persistent organochloride pesticides cause reproductive and developmental toxicity are not currently known in detail, but it is most likely that these compounds act through different mechanisms. Therefore, both *in vitro* and *in vivo* bioassays should be used in further research to understand these mechanistic pathways.

Abbreviations

DON: Deoxynivalenol; OTA: ochratoxin A; α -ZOL: alpha-zearalenol; β -ZOL: beta-zearalenol; *p,p'*-DDT: 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane; *p,p'*-DDE: 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene; *o,p'*-DDT: 1,1,1-trichloro-2(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethane; EDCs: endocrine disrupting chemicals; PBS: Dulbecco's phosphate buffered saline; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution; SDS: sodium dodecyl sulfate; HCl: hydrochloric acid; DMEM/F-12: Dulbecco's Modified Eagle Medium/F-12 nutrient mixture; ANOVA: analysis of variance; DMSO: dimethyl sulfoxide; E₂: oestradiol/17 β -oestradiol; RGA: Reporter gene assay; ER: oestrogen receptor; ESR1 (ER α): oestrogen receptors alpha; ESR1 (ER α): oestrogen receptors beta; ATCC: American type culture collection; CALUX: Chemically activated luciferase expression; DBP: di-*n*-butyl phthalate; PB–Bu: butyl 4-hydroxybenzoate; and PB–Pr: propyl 4-hydroxybenzoate

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Appendix

Supplementary file 1. Cell viability of MMV-Luc cell line exposed to different chemical treatments.

Supplementary file 2. The interactive effects of combined mycotoxins and/or persistent organochloride pesticides on E₂-mediated ER transcriptional activation.

References

Akoto, O., Andoh, H., Darko, G., Eshun, K., Osei-Fosu, P., 2013. Health risk assessment of pesticides residue in maize and cowpea from Ejura, Ghana. *Chemosphere* 92(1), 67-73.

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F.A., Oswald, I.P., Puel, O., 2013. New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. *Toxicol Appl Pharmacol.* 272(1), 191-198.

Al-Bader, M., Ford, C., Al-Ayadhy, B. and Francis, I., 2011. Analysis of estrogen receptor isoforms and variants in breast cancer cell lines. *Exp Ther Med.* 2(3), 537-544.

Ayed, Y., Ayed-Boussema, I., Ouanes, Z., Bacha, H., 2011. In vitro and in vivo induction of chromosome aberrations by alpha-and beta-zearalenols: Comparison with zearalenone. *Mutat Res Genet Toxicol Environ Mutagen.* 726(1), 42-46.

Balaguer, P., Joyeux, A., Denison, M.S., Vincent, R., Gillesby, B.E., Zacharewski, T., 1996. Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using in vitro recombinant receptor reporter gene assays. *Can J Physiol Pharmacol.* 74(2), 216-222.

Bardin, A., Boulle, N., Lazennec, G., Vignon, F. and Pujol, P., 2004. Loss of ER β expression as a common step in estrogen-dependent tumor progression. *Endocr.-Relat. Cancer* 11(3), 537-551.

Bittner, G.D., Denison, M.S., Yang, C.Z., Stoner, M.A., He, G., 2014. Chemicals having estrogenic activity can be released from some bisphenol a-free, hard and clear, thermoplastic resins. *Environ. Health* 13(1), 103.

Bowers, J.L., Tyulmenkov, V.V., Jernigan, S.C. and Klinge, C.M., 2000. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β . *Endocrinology* 141(10), 3657-3667.

Brandenberger, A.W., Tee, M.K., Lee, J.Y., Chao, V. and Jaffe, R.B., 1997. Tissue distribution of estrogen receptors alpha (ER- α) and beta (ER- β) mRNA in the midgestational human fetus. *J Clin Endocrinol Metab.* 82(10), 3509-3512.

Brandenberger, A.W., Tee, M.K. and Jaffe, R.B., 1998. Estrogen receptor alpha (ER- α) and beta (ER- β) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER- β in neoplastic tissues. *J Clin Endocrinol Metab.* 83(3), 1025-1028.

Brandenberger, A.W., Lebovic, D.I., Tee, M.K., Ryan, I.P., Tseng, J.F., Jaffe, R.B. and Taylor, R.N., 1999. Oestrogen receptor (ER)- α and ER- β isoforms in normal endometrial and endometriosis-derived stromal cells. *Mol Human Reprod.* 5(7), 651-655.

Brennan, J.C., Bassal, A., He, G., Denison, M.S., 2016. Development of a recombinant human ovarian (BG1) cell line containing estrogen receptor α and β for improved detection of estrogenic/antiestrogenic chemicals. *Environ Toxicol Chem.* 35(1), 91-100.

Chen, C.W., Hurd, C., Vorojeikina, D.P., Arnold, S.F., Notides, A.C., 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. *Biochem Pharmacol.* 53, 1161-1172.

Clarke, R., Connolly, L., Frizzell, C., Elliott, C.T., 2014. Cytotoxic assessment of the regulated, co-existing mycotoxins aflatoxin B1, fumonisin B1 and ochratoxin, in single, binary and tertiary mixtures. *Toxicon* 90, 70-81.

Connolly, L., 2009. Endocrine disrupting chemicals: origins, fates and transmission into the food chain. *Endocrine Disrupting Chemicals in Food.* Woodhead Publishing Ltd, Cambridge, England, pp.103-125.

Connolly, L., Ropstad, E., Verhaegen, S., 2011. In vitro bioassays for the study of endocrine-disrupting food additives and contaminants. *Trends Anal Chem.* 30, 227-238.

Cozzini, P., Dellaflora, L., 2012. In silico approach to evaluate molecular interaction between mycotoxins and the estrogen receptors ligand binding domain: a case study on zearalenone and its metabolites. *Toxicol Lett.* 214(1), 81-85.

Dänicke, S., Winkler, J., 2015. Invited review: Diagnosis of zearalenone (ZEN) exposure of farm animals and transfer of its residues into edible tissues (carry over). *Food Chem Toxicol.* 84, 225-249.

Demaegdt, H., Daminet, B., Evrard, A., Scippo, M.L., Muller, M., Pussemier, L., Callebaut, A., Vandermeiren, K., 2016. Endocrine activity of mycotoxins and mycotoxin mixtures. *Food Chem Toxicol.* 96, 107-116.

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev.* 30(4), 293-342.

Drzymala, S.S., Binder, J., Brodehl, A., Penkert, M., Rosowski, M., Garbe, L.A., Koch, M., 2015. Estrogenicity of novel phase I and phase II metabolites of zearalenone and cis-zearalenone. *Toxicon* 105, 10-12.

Dua V.K., Kumari R, Sharma V.P, Subbarao S.K., 2001. Organochlorine residues in human blood from Nainital (UP), India. *Bull. Environ Contam Toxicol.* 67, 42-45.

Ehrlich, V.A., Dellafiora, L., Mollergues, J., Dall'Asta, C., Serrant, P., Marin-Kuan, M., Lo Piparo, E., Schilter, B., Cozzini, P., 2015. Hazard assessment through hybrid in vitro/in silico approach: The case of zearalenone. *Altex* 32(4), 275.

Elmlinger, M.W., Kühnel, W., Ranke, M.B., 2002. Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults. *Clin Chem Lab Med.* 40(11), 1151-1160.

European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM)., 2016. Appropriateness to set up a group health-based guideline value for zearalenone and its modified forms. *EFSA Journal* 14, 4425-4471. Doi: 10.2903/j.efsa.2016.4425

EFSA CONTAM Panel (European Food Safety Authority Panel on Contaminants in the Food Chain), Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., 2017. Scientific Opinion on the risks for animal health related to the presence of zearalenone and its modified forms in feed. *EFSA Journal* 15(7), 4851. <https://doi.org/10.2903/j.efsa.2017.4851>

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

Frizzell, C., Verhaegen, S., Ropstad, E., Elliott, C.T., Connolly, L., 2013. Endocrine disrupting effects of ochratoxin A at the level of nuclear receptor activation and steroidogenesis. *Toxicol Lett.* 217(3), 243-250.

Grenier, B., Oswald, I., 2011. Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. *World Mycotoxin J.* 4(3), 285-313.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P., 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect.* 103(6), 582-587.

Joint FAO/WHO Expert Committee on Food Additives (JECFA)., 2000. Toxicological evaluation of certain veterinary drug residues in food: estradiol-17 β , progesterone, and testosterone. WHO food additives series 43. World Health Organization, Geneva. <http://www.inchem.org/documents/jecfa/jecmono/v43jec05.htm> [Accessed 29/10/2017].

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A., Wilson, E.M., 1995. Persistent DDT metabolite p, p'-DDE is a potent androgen receptor antagonist. *Nature* 375(6532), 581-585.

Kelce, W.R., Wilson, E.M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. *J Mol Med.* 75(3), 198-207.

Klein-Hitpass, L., Ryffel, G.U., Heitlinger, E. and Cato, A.C., 1988. A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. *Nucleic Acids Res.* 16(2), 647-663.

Kowalska, K., Habrowska-Górczyńska, D.E., Piastowska-Ciesielska, A.W., 2016. Zearalenone as an endocrine disruptor in humans. *Environ Toxicol Pharmacol.* 48, 141-149.

Kuiper, G.G., Lemmen, J.G., Carlsson, B.O., Corton, J.C., Safe, S.H., Van Der Saag, P.T., Van Der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* 139(10), 4252-4263.

Legler, J., van den Brink, C.E., Brouwer, A., Murk, A.J., van der Saag, P.T., Vethaak, A.D., van der Burg, B., 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol Sci.* 48(1), 55-66.

Li, J., Li, N., Ma, M., Giesy, J.P., Wang, Z., 2008. In vitro profiling of the endocrine disrupting potency of organochlorine pesticides. *Toxicol lett.* 183(1-3), 65-71.

Longnecker, M.P., Klebanoff, M.A., Brock, J.W., Zhou, H., Gray, K.A., Needham, L.L., Wilcox, A.J., 2002. Maternal serum level of 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene and risk of cryptorchidism, hypospadias, and polythelia among male offspring. *Am J Epidemiol.* 155, 313-322.

Massart, F., Meucci, V., Saggese, G., Soldani, G., 2008. High growth rate of girls with precocious puberty exposed to oestrogenic mycotoxins. *J Pediatr.* 152, 690-695.

Metzler, M., Pfeiffer, E., Hildebrand, A., 2010. Zearalenone and its metabolites as endocrine disrupting chemicals. *World Mycotoxin J.* 3(4), 385-401.

Minervini, F., Dell'Aquila, M. E., 2008. Zearalenone and Reproductive Function in Farm Animals. *Int J Mol Sci.* 9, 2570-2584.

Molina-Molina, J.M., Real, M., Jimenez-Diaz, I., Belhassen, H., Hedhili, A., Torné, P., Fernández, M.F., Olea, N., 2014. Assessment of estrogenic and anti-androgenic activities of the mycotoxin zearalenone and its metabolites using in vitro receptor-specific bioassays. *Food Chem Toxicol.* 74, 233-239.

Mosselman, S., Polman, J. and Dijkema, R., 1996. ER β : identification and characterization of a novel human estrogen receptor. *FEBS lett.* 392(1), 49-53.

Mueller, S.O., Simon, S., Chae, K., Metzler, M. and Korach, K.S., 2004. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ER α) and ER β in human cells. *Toxicol Sci.* 80(1), 14-25.

Ndossi, D.G., Frizzell, C., Tremoen, N.H., Fæste, C.K., Verhaegen, S., Dahl, E., Eriksen, G.S., Sørli, M., Connolly, L., Ropstad, E., 2012. An in vitro investigation of endocrine disrupting effects of trichothecenes deoxynivalenol (DON), T-2 and HT-2 toxins. *Toxicol lett.* 214(3), 268-278.

Pillay, D., Chuturgoon, A.A., Nevines, E., Manickum, T., Deppe, W., Dutton, M.F., 2002. The quantitative analysis of zearalenone and its derivatives in plasma of patients with breast and cervical cancer. *Clin Chem Lab Med.* 40(9), 946-951.

Pillon, A., Boussioux, A.M., Escande, A., Aït-Aïssa, S., Gomez, E., Fenet, H., Ruff, M., Moras, D., Vignon, F., Duchesne, M.J., Casellas, C., 2005. Binding of estrogenic compounds to recombinant estrogen receptor- α : application to environmental analysis. *Environ Health Perspect.* 113(3), 278-284.

Rodrigues, I., Naehrer, K., 2012. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. *Toxins* 4(9), 663-675.

Romero-González, R., Frenich, A.G., Vidal, J.M., Prestes, O.D., Grió, S.L., 2011. Simultaneous determination of pesticides, biopesticides and mycotoxins in organic products applying a quick, easy, cheap, effective, rugged and safe extraction procedure and ultra-high performance liquid chromatography–tandem mass spectrometry. *J Chromatogr A.* 1218(11), 1477-1485.

Shephard, G. S., Burger, H-S, Gambacorta, L., Gong, Y. Y., Krska, R., Rheeder, J. P., Solfrizzo, M., Srey, C., Sulyok, M., Visconti, A., Warth, B., van der Westhuizen, L., 2013. Multiple mycotoxin exposure determined by urinary biomarkers in rural subsistence farmers in the former Transkei, South Africa. *Food Chem Toxicol.* 62, 217-225.

Shier, W.T., Shier, A.C., Xie, W., Mirocha, C.J., 2001. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicon* 39(9), 1435-1438.

Sonneveld, E., Jansen, H.J., Riteco, J.A., Brouwer, A., van der Burg, B., 2005. Development of androgen-and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicol Sci.* 83(1), 136-148.

Sotoca Covalada, A.M., Van den Berg, H., Vervoort, J., Van der Saag, P., Ström, A., Gustafsson, J.Å., Rietjens, I. and Murk, A.J., 2008. Influence of cellular ER α /ER β ratio on the ER α -agonist induced proliferation of human T47D breast cancer cells. *Toxicol Sci.* 105(2), 303-311.

Stypuła-Trębas, S., Minta, M., Radko, L., Żmudzki, J., 2016. Oestrogenic and (anti) androgenic activity of zearalenone and its metabolites in two in vitro yeast bioassays. *World Mycotoxin J.* 9(2), 247-255.

Tatay, E., Espín, S., García-Fernández, A.J., Ruiz, M.J., 2018. Estrogenic activity of zearalenone, α -zearalenol and β -zearalenol assessed using the E-screen assay in MCF-7 cells. *Toxicol Mech Methods.* 28(4), 239-242.

Vejdovszky, K., Hahn, K., Braun, D., Warth, B., Marko, D., 2017a. Synergistic estrogenic effects of *Fusarium* and *Alternaria* mycotoxins in vitro. *Arch Toxicol.* 91(3), 1447-1460.

Vejdovszky, K., Schmidt, V., Warth, B., Marko, D., 2017b. Combinatory estrogenic effects between the isoflavone genistein and the mycotoxins zearalenone and alternariol in vitro. *Mol Nutr Food Res.* 61(3), 1600526.

Wang, S., Aarts, J.M., Evers, N.M., Peijnenburg, A.A., Rietjens, I.M., Bovee, T.F., 2012. Proliferation assays for estrogenicity testing with high predictive value for the in vivo uterotrophic effect. *J Steroid Biochem Mol Biol.* 128(3-5), 98-106.

Wang, S., Rijk, J.C., Besselink, H.T., Houtman, R., Peijnenburg, A.A., Brouwer, A., Rietjens, I.M., Bovee, T.F., 2014a. Extending an in vitro panel for estrogenicity testing: the added value of bioassays for measuring antiandrogenic activities and effects on steroidogenesis. *Toxicol Sci.* 141(1), 78-89.

Wang, S., Aarts, J.M., Haan, L.H., Argyriou, D., Peijnenburg, A.A., Rietjens, I.M., Bovee, T.F., 2014b. Towards an integrated in vitro strategy for estrogenicity testing. *J Appl Toxicol.* 34(9), 1031-1040.

Warth, B., Parich, A., Atehnkeng, J., Bandyopadhyay, R., Schuhmacher, R., Sulyok, M., Krska, R., 2012. Quantitation of mycotoxins in food and feed from Burkina Faso and Mozambique using a modern LC-MS/MS multitoxin method. *J Agric Food Chem.* 60(36), 9352-9363.

Warth, B., Sulyok, M., Krska, R., 2013. LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxins. *Anal Bioanal Chem.* 405(17), 5687-5695.

Weber, F., Freudinger, R., Schwerdt, G. and Gekle, M., 2005. A rapid screening method to test apoptotic synergisms of ochratoxin A with other nephrotoxic substances. *Toxicol In Vitro.* 19(1), 135-143.

Wielogórska, E., Elliott, C.T., Danaher, M., Connolly, L., 2015. Endocrine disruptor activity of multiple environmental food chain contaminants. *Toxicol In Vitro.* 29(1), 211-220.

Willemsen, P., Scippo, M.L., Kausel, G., Figueroa, J., Maghuin-Rogister, G., Martial, J.A., Muller, M., 2004. Use of reporter cell lines for detection of endocrine-disrupter activity. *Anal Bioanal Chem.* 378(3), 655-663.

Wilson, V.S., Bobseine, K. and Gray Jr, L.E., 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci.* 81(1), 69-77.

Fig. 1: Dose response curve of the transcriptional response elicited by 17 β -oestradiol (E₂), zearalenone (ZEN), alpha-zearalenol (α -ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) with MMV-Luc reporter gene cell line. Test substances were assayed in triplicate in three independent experiments. Error bars represent standard deviation from three biological replicates. Results are expressed as percentage of maximal induction of E₂ (100%; 10 nM).

Fig. 2. Transcriptional response induced when increasing concentrations of single mycotoxins and persistent organochloride pesticides were co-exposed with either 0.05 nM or 10 nM of E₂. Test substances were assayed in triplicate, in at least two independent experiments. Error bars represent standard deviation from at least two biological replicates. Results are expressed as percentage of maximal induction of E₂ (0.05 nM or 10 nM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. 3. Transcriptional response induced when increasing concentrations of binary mixtures of mycotoxins and persistent organochloride pesticides were co-exposed with either 0.05 nM or 10 nM of E₂. Test substances were assayed in triplicate, in at least two independent experiments. Error bars represent standard deviation from at least two biological replicates. Results are expressed as percentage of maximal induction of E₂ (0.05 nM or 10 nM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. 4. Transcriptional response induced when increasing concentrations of binary mixtures of α -ZOL and persistent organochloride pesticides (p,p' -DDT and p,p' -DDE) were co-exposed with either 0.05 nM or 10 nM of E_2 . Test substances were assayed in triplicate, in at least two independent experiments. Error bars represent standard deviation from at least two biological replicates. Results are expressed as percentage of maximal induction of E_2 (0.05 nM or 10 nM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Table 1: The EC₅₀ and maximal ER transcriptional response of the tested mycotoxins and pesticides

	E₂	ZEN	<i>α</i>-ZOL	<i>p,p'</i>-DDT	<i>p,p'</i>-DDE
EC₅₀	5.30 x 10 ⁻¹¹ ± 1.17 x 10 ⁻¹¹ M (0.053 ± 0.012 nM)	1.32 x 10 ⁻⁹ ± 9.56 x 10 ⁻¹¹ M (1.32 ± 0.0956 nM)	2.70 x 10 ⁻¹⁰ ± 1.21 x 10 ⁻¹⁰ M (0.27 ± 0.121 nM)	N/A	N/A
R²	0.9952	0.9905	0.9888	0.9845	0.9895
Maximal induction (%)	100 ± 0.00	80.11 ± 5.58	73.45 ± 3.25	28.70 ± 2.97	18.65 ± 1.77

SUPPLEMENTARY INFORMATION

The effect of single and mixtures of mycotoxins and persistent organochloride pesticides on oestrogen receptor transcriptional activation using *in vitro* reporter gene assays

Ukpai A. Eze^{a, b, c, ψ}, John D. Huntriss^c, Michael N. Routledge^d, and Yun Yun Gong^{a, e, *} and

Lisa Connolly^f

Food and Chemical Toxicology

Supplementary file 1. Cell viability of MMV-Luc cell line exposed to different chemical treatments

Fig. S1.1: Cell viability of MMV-Luc reporter gene cell line exposed to 17 β -oestradiol (E₂), zearalenone (ZEN), alpha-zearalenol (α -ZOL), 1,1,1-trichloro-2,2-bis(**p**-chlorophenyl) ethane (p,p' -DDT), and 1,1-dichloro-2,2-bis(**p**-chlorophenyl) ethylene (p,p' -DDE). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error

bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of methanol (MeOH) control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. S1.2: Cell viability of MMV-Luc reporter gene cell line exposed to either zearalenone (ZEN) or alpha-zearalenol (α -ZOL) in combination with 0.05 nM or 10 nM of 17 β -oestradiol (E₂). Test substances were assayed in triplicate in three independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of three biological replicates. Results are expressed as percentage of methanol (MeOH) control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. S1.3: Cell viability of MMV-Luc reporter gene cell line exposed to either 1,1,1-trichloro-2,2-bis(**p**-chlorophenyl) ethane (*p,p'*-DDT), and 1,1-dichloro-2,2-bis(**p**-chlorophenyl) ethylene (*p,p'*-DDE) in combination with 0.05 nM or 10 nM of 17β-oestradiol (E₂). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of methanol (MeOH) control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. S1.4: Cell viability of MMV-Luc reporter gene cell line exposed to binary combination of zearalenone (ZEN) with either alpha-zearalenol (α -ZOL), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (*p,p'*-DDT), or 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDE) in conjunction with 0.05 nM or 10 nM of 17 β -oestradiol (E₂). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of maximal induction of E₂ (0.05 nM or 10 nM). Results are expressed as percentage

of methanol (MeOH) control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. S1.5: Cell viability of MMV-Luc reporter gene cell line exposed to binary combination of α -ZOL with either p,p' -DDT (A & B), or p,p' -DDE (C & D), and co-exposure of p,p' -DDT and p,p' -DDE (E & F) in conjunction with 0.05 nM or 10 nM of 17 β -oestradiol (E₂). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as

percentage of maximal induction of E₂ (0.05 nM or 10 nM). Results are expressed as percentage of methanol (MeOH) control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

SUPPLEMENTARY INFORMATION

The effect of single and mixtures of mycotoxins and persistent organochloride pesticides on oestrogen receptor transcriptional activation using *in vitro* reporter gene assays

Ukpai A. Eze^{a, b, c, ψ}, John D. Huntriss^c, Michael N. Routledge^d, and Yun Yun Gong^{a, e, *} and

Lisa Connolly^f

Food and Chemical Toxicology

Supplementary file 2. The interactive effects of combined mycotoxins and/or persistent organochloride pesticides on E₂-mediated ER transcriptional activation

Fig. S2.1: Measured and expected ER transcriptional response induced when increasing concentrations of binary mixtures of mycotoxins and persistent organochloride pesticides were co-exposed with either 0.05 nM or 10 nM of E₂. Test substances were assayed in triplicate, in three independent experiments. Data were analysed with multiple t-test and corrected for multiple comparison using the Holm-Šidák test method. Error bars represent standard deviation

of three biological replicates. Results are expressed as percentage of maximal induction of E₂ (0.05 nM or 10 nM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.

Fig. S2.2: Measured and expected ER transcriptional response induced when increasing concentrations of binary mixtures of α -ZOL and persistent organochloride pesticides (p,p' -

DDT and *p,p'*-DDE) were co-exposed with either 0.05 nM or 10 nM of E₂. Test substances were assayed in triplicate in three independent experiments. Data were analysed with multiple t-test and corrected for multiple comparison using the Holm-Šídák test method. Error bars represent standard deviation of three biological replicates. Results are expressed as percentage of maximal induction of E₂ (0.05 nM or 10 nM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ represents significant effects.