AN *IN OVO* TOXICOLOGICAL ASSESSMENT OF INDIVIDUAL AND COMBINED *FUSARIUM* MYCOTOXINS IN THE CHICKEN EMBRYO

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ABSTRACT

The increasing occurrence of *Fusarium* fungi and associated mycotoxins in cereal grains is a significant issue for global agriculture. The mycotoxin deoxynivalenol (DON) is the most prevalent feed contaminant worldwide and causes a variety of adverse effects in animals. While the individual toxicity of DON is a concern, exposure to multiple mycotoxins in feed is more common, necessitating information on the outcome of mixture exposures. However, characterizing the toxicity of DON and other mycotoxins has been difficult due to highly varied responses in long-term animal feeding trials. In addition, resources required for whole animal testing are only amplified in combinatorial mycotoxin studies given the large sample sizes and number of treatment groups required. The chicken embryo has been widely and successfully utilized as a non-animal alternative to evaluate the toxicity of environmental pollutants and could be used as a screening tool to evaluate mycotoxin mechanisms of action and mixture toxicity. The overall objective of this thesis research is to characterize the effects of DON (administered *in ovo*) alone and in combination with a commonly co-occurring mycotoxins, zearalenone (ZEA), to the late-term chicken embryo in order to determine whether an *in ovo* approach for conducting exposures to *Fusarium* mycotoxins could be used as a predictive tool for assessing the toxicity of *Fusarium* mycotoxins alone and in combination. The overall hypothesis is that responses of the late-term chicken embryo to single doses of *Fusarium* mycotoxins, alone or in combination, are similar to those reported in whole animal feeding trials with poultry.

In the first experiment, the effect of *in ovo* administration of DON was evaluated in terms of embryotoxicity, growth and development, pathological changes to tissue, and biochemical/molecular indicators of oxidative and immune stress. A single injection of purified DON was administered to the late-term chicken embryo (embryonic day 14, ED14) at five doses ranging from 0.0 – 5.0 μg DON/g egg weight. Eggs were opened on ED20 and embryos were evaluated for survivability and growth parameters. Tissues were sampled for subsequent analysis. At the highest dose, DON decreased embryo survivability and increased the absolute and relative weight of both liver and spleen. Hepatic bile stagnation and concurrent splenic inflammation were frequently detected among groups receiving 5.0 and 1.0 μg DON/g egg weight but were observed less often in the latter. A dose-dependent increase in granulopoiesis and lipid peroxidation (as measured by TBARS assay) were observed in the liver; however, mRNA expression of genes related to immune and oxidative stress were mostly unchanged. These results suggest that the chicken embryo responds to *in ovo* DON exposure with effects on

immunity and oxidative stress that are supported by previous *in vivo* and *in vitro* findings. The *in ovo* approach developed and validated in the first experiment was then carried forward to a second experiment with the aim of characterizing the combined toxicity of DON and another mycotoxin, ZEA to the chicken embryo. ZEA was chosen for this experiment because the combination of DON and ZEA is considered to be the most prevalent mycotoxin mixture in North America and worldwide. Treatments included an untreated control group (CON), a vehicle-injected control group (20% DMSO), 0.5 and 2.5 µg DON/g egg weight, 0.5 and 2.5 µg ZEA/g egg weight, and a low and high combination treatment at 0.5 µg DON + 0.5 µg ZEA/g egg weight and 2.5 µg DON + 2.5 µg ZEA/g egg weight, respectively. The results demonstrated that interactive effects of DON and ZEA differed across endpoints and tended to vary from antagonistic at low doses to non-interactive or possibly potentiated at high doses. At low doses, DON and ZEA had antagonistic effects on liver weight as well as liver lipid peroxidation. At high doses, effects of DON and ZEA were mostly independent and effects of DON, specifically, were in line with our previous observations. At a combined, high dose of DON and ZEA there was evidence of possible potentiation with respect to embryo survivability, hepatic bile stagnation and splenic inflammation, and hepatic granulopoiesis. These results suggest the chicken *in ovo* model is useful for studying combinatorial mycotoxin toxicity; however, further research regarding ZEA-induced toxicity would improve response interpretation. Overall, the results presented in this thesis indicate that *in ovo* responses to *Fusarium* mycotoxins, alone and in combination, are supported by previous *in vitro* and *in vivo* findings. While *in ovo* mycotoxin exposures cannot replace *in vivo* experimentation, there is potential for the *in ovo* model to inform whole animal studies by identifying and prioritizing emerging mycotoxins and high-risk mycotoxin combinations for further *in vivo* assessment. In the future, the *in ovo* model could be used in a more practical application as a rapid-screening tool to assess the toxicity of mycotoxin grain extracts or to evaluate the efficacy of new mycotoxin mitigation techniques.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

18S	18S ribosomal ribonucleic acid
AF	aflatoxin
AFB	aflatoxin B_1
AIFM1	apoptosis-inducing factor 1, mitochondrial
BAX	Bcl-2-associated X protein
CA	California
CASP	caspase
cDNA	complementary deoxyribonucleic acid
CFIA	Canadian Food Inspection Agency
CGC	Canadian Grains Commission
DE	Delaware
DON	deoxynivalenol
DMSO	dimethyl sulfoxide
ED	embryonic day
EU	European Union
FCR	Fusarium Crown Rot
FDA	Food and Drug Administration
FHB	Fusarium Head Blight
FUM	fumonisin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
g/day	grams per day
GPx	glutathione peroxidase
GSH	glutathione
H & E	hematoxylin and eosin

IgA	immunoglobulin A
IL	interleukin
MB	Manitoba
MA	Massachusetts
µg/g	microgram per gram
µL/g	microliter per gram
mg/kg	milligram per kilogram
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MO	Missouri
mRNA	mitochondrial ribonucleic acid
NC	North Carolina
nm	nanometer
ON	Ontario
OTA	ochratoxin
ppm	parts per million
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SAS	Statistical Analysis Software
TBARS	thiobarbituric acid reactive substances
TX	Texas
UK	United Kingdom
USA	United States of America
VT	Vermont
YFBW	yolk-free body weight
ZEA	zearalenone

ZEL	zearalenol
α	alpha
β	beta

NOTE TO READER

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Therefore, there is some repetition between the material presented in each chapter. Chapter 1 of this thesis is a review of the current literature as it pertains to mycotoxin occurrence, individual and combined toxicity of mycotoxins with special reference to poultry, the applicability of *in ovo* exposures for evaluating mycotoxin toxicity and concludes with research hypothesis and objectives. Chapter 4 is a general discussion containing major conclusions of the thesis research. Chapter 2 and Chapter 3 are organized as manuscripts for publication in peer-reviewed scientific journals and a description of author contributions is provided following the preface for these chapters. References cited in each chapter are combined and listed in the References section of the thesis.

CHAPTER 1

LITERATURE REVIEW

1.1 *Fusarium* fungi and associated mycotoxins in Canada

The infection of cereal grains by *Fusarium* fungi is an increasingly prevalent issue for global agriculture. *Fusarium* fungal diseases compromise the quality and yield of crops while accumulation of toxic fungal metabolites, known as mycotoxins, within the grains themselves makes them unsafe for human and animal consumption. The two main diseases generated by *Fusarium* infection in cereals are Fusarium Head Blight (FHB) and Fusarium Crown Rot (FCR), both of which are observed in cereal crops like wheat, barley, rye, oats and triticale (Fouroud et al., 2014). The major species responsible for FCR is *Fusarium pseudograminearnum* and although occasionally found in Canada, this species tends to prefer warmer climates (Miller and Richardson, 2013). More relevant to Canadian production is the disease FHB and its causative agents, *Fusarium graminearum* and *Fusarium culmorum*. These species are known to be highly pathogenic and abundant and under stressful conditions (i.e. nutritional imbalance, drought, water excess etc.), can produce mycotoxins that accumulate in grains (Rodrigues and Naehrer, 2012) – primarily deoxynivalenol (DON; Figure 1.1: A) and zearalenone (ZEA; Figure 1.1: B) in cooler climates (Schatzmayr and Streit, 2013). These mycotoxins are likely the most relevant to Canadian production and confer the most risk to human and animal health. Although not as acutely toxic as some mycotoxins, DON is the most common and abundant mycotoxin in Canada and throughout the world (Rodrigues and Naehrer, 2012). Tittlemier et al. (2013) quantified DON in 75% of Canadian western amber durum samples obtained in the 2010 Canadian Grains Commission Harvest Sample Program. A recent global mycotoxin survey reported that, in North America, DON and ZEA contaminated 83% and 42% of finished feed samples, respectively (Biomin, 2017). Similar to DON, ZEA is not considered acutely toxic but behaves estrogenically and thus, is biologically potent.

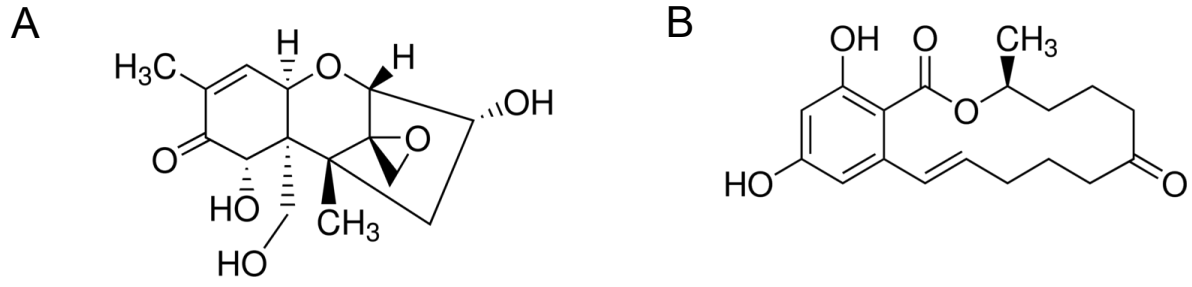

Figure 1.1 The chemical structure of the *Fusarium* mycotoxins deoxynivalenol (DON; A) and zearalenone (ZEA; B) (Sigma–Aldrich Corporation, St. Louis, MO, USA).

Mycotoxin concentration is a significant factor in the grading of wheat and durum and with 75% of Canadian wheat exported, mycotoxin contamination confers significant economic losses to the agricultural sector (Tittlemier et al., 2013). Within the United States, estimated total economic loss due to mycotoxin contamination average $932 million per year (CAST report, 2003). The Food and Agricultural Organization (FAO) estimates 25% of the world's crops are affected by mycotoxins and that food and food product losses near 1 billion metric tons (2007). Crop losses due to mycotoxin contamination will increase globally in the future as incidence of mycotoxins in grain crops continues to rise.

In Canada, conditions favoring the development of *Fusarium* infection have been, in part, attributed to increasing mycotoxin levels. It is well known that temperature and precipitation around the time of flowering (anthesis) are important factors in *Fusarium* infection of cereals, such as wheat (Bailey et al., 2003), and high levels of mycotoxin contamination are linked to unusual weather (Schatzmayr and Streit, 2013). While the climate of western Canada has historically been effective at hindering *Fusarium* species, increasing yearly moisture during anthesis has promoted fungal spread and thus, the production of mycotoxins. Moreover, the genetics of *Fusarium* species on the prairies is shifting and, in turn, altering mycotoxin matrices in grains. The fusario-mycotoxin DON is produced by *Fusarium* via two precursor pathways: 15-acetyl DON (15-ADON) and 3-acetyl DON (3-ADON). Isolates from the 3-ADON chemotype have been shown to produce significantly more DON per kernel and have increased growth rates as compared to 15-ADON (Ward et al., 2008). Historically, the 15-ADON chemotype has dominated in western Canada; however, data shows that the 3-ADON chemotype is becoming increasing prevalent (Canadian Grains Commission, 2008). This shift ultimately results in a higher overall concentration of DON within *Fusarium* infected grains.

1.1.1 Regulating mycotoxins in livestock feed

Currently, many regulatory bodies (*i.e.* Canadian Food Inspection Agency, CFIA; Food and Drug Administration, FDA; European Union; EU) impose limits on the concentration of mycotoxins permitted in animal feed. Various factors contribute to the establishment of mycotoxin limits including scientific, economic, or political factors (van Egmond, 2002); thus, maximum inclusion levels vary across countries. In Canada, only a small proportion of all mycotoxins are subject to limitation and these include aflatoxins (AFs), ochratoxin (OTA), fumonisins (FUM), diacetoxyscirpenol, ergot, T-2 toxin, HT-2 toxin as well as DON and ZEA

(CFIA, 2015). Compliance with limits is ensured by regular testing of grains along the grain handling and processing chain (Foroud et al., 2014) – domestic and export grain shipments are monitored by the Canadian Grains Commission (CGC) while designated feed commodities are monitored by the CFIA. However, it is important to note that only levels of aflatoxin in feed are actually regulated while all other existing limits are recommended guidelines (CFIA, 2015). Whether recommended or legislated, maximum levels for mycotoxins are designed to prevent in-feed mycotoxin concentrations that cause mycotoxicosis or reduced growth performance in livestock, and/or to prevent carry-over of mycotoxins to animal products designated for human consumption. Regulatory bodies such as the CFIA defines those maximums based on the sensitivity of species to individual mycotoxins. What current limits in Canada, and worldwide, do not consider is that feed commodities are often co-contaminated by mycotoxins (Smith et al., 2016) and interactions between mycotoxins in the feed matrix are likely to increase their toxic effect (Grenier and Oswald, 2011). This limitation can mainly be attributed to the lack of information regarding interactive toxicity of mycotoxins (Smith et al., 2016). While it would be nearly impossible to characterize the toxicity of every mycotoxin combination for each livestock species, identification and characterization of particularly high-risk combinations would provide relevant information to the livestock industry.

1.1.2 Co-occurrence of mycotoxins

Co-occurrence of mycotoxins can be explained by the fact that 1) most *Fusarium* fungi produce several mycotoxins concurrently, 2) several fungi can contaminate feed commodities simultaneously or in quick succession, and 3) animal diets are usually made up of multiple grain sources (Smith et al., 2016). A recent, world-wide survey of feeds and raw feed ingredients identified more than one mycotoxin in 71% of 18,757 samples tested (Biomin, 2017). In North America, specifically, a similar survey reported that 48% of raw feed ingredients and 40% of finished feeds in North America contained two or more mycotoxins (Rodrigues and Naehrer, 2012). The most commonly detected co-occurring mycotoxins include *Fusarium* toxins (namely type B-trichothecenes (like DON), ZEA, and FUM), AFs and OTA, in various combinations (Streit et al., 2012). Indeed, in their meta-analyses, Smith et al. (2016) identified binary combinations of AFs+FUM, AFs+OTA, FUM+ZEA and DON+ZEA as the most frequently observed in cereals and cereal product samples. This analyses also concluded that the

combination of DON + ZEA was the most prevalent mycotoxin combination in North America (Smith et al. 2016).

As discussed above, current regulations do not reflect the combinatorial nature of mycotoxins, despite the frequency of mycotoxin co-contamination in feed, as data on combined toxicity of mycotoxins are varied and limited (Smith et al., 2016). Generally, we can see that naturally contaminated feed has a more potent effect in terms of toxicity when compared to feed that is spiked with a single pure mycotoxin (reviewed in Eriksen and Petterson, 2004). This discrepancy is often attributed to the presence of masked, previously undescribed or co-occurring mycotoxins, that have not been tested for, acting in sync with known mycotoxins (Schatzmayr and Streit, 2013; Dersjant-Li et al., 2003; Trenholm et al., 1994). Similarly, co-occurring mycotoxins can interact in various ways and these interactions can be defined as additive, synergistic, permissive (or potentiated), or antagonistic. Regarding effects on animal performance, most studies report additive or synergistic interactions of co-occurring mycotoxins (Grenier and Oswald, 2011). This means that co-contaminated samples with individual concentrations even below those recommended by regulatory bodies may still exert effects due to synergistic interactions of the mycotoxins (Schatzmayr and Streit, 2013), explaining in part why animals sometimes react adversely to feed, despite compliant mycotoxin levels (Rodrigues and Naehrer, 2012).

Evaluating the adverse effects of mycotoxin mixtures provides more relevant information to the livestock industry; however, as previously mentioned, there is limited information available in this context, particularly in the whole animal. Several authors have identified the need for combinatorial mycotoxin research to support improved in-feed guidelines (Speijers and Speijers, 2004; Grenier and Oswald, 2011; Streit et al., 2012; Smith et al., 2016). While many studies exist on the individual toxicity of mycotoxins, in practice, the outcome of combined exposure may be qualitatively or quantitatively different from what would be predicted based on individual toxicity (Speijers and Speijers, 2004).

1.2 Mechanisms of Action

1.2.1 Deoxynivalenol (DON)

Toxicity of DON is conferred primarily through potent inhibition of protein synthesis and induction of apoptosis (Rotter et al., 1996). Maresca et al. (2002) showed that effects of DON

were mimicked by protein synthesis inhibitors and apoptosis inducers and suggested that inhibition of protein synthesis and induction of apoptosis are the main mechanisms of DON toxicity. These actions are accomplished by the interaction of DON with cellular targets. Within the cell, DON binds the 60S ribosomal subunit and exerts translational inhibition (Ueno, 1984), while also rapidly activating mitogen-activated protein kinases (MAPKs) including p38, c-Jun N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK) (Shifrin and Anderson, 1999). Cascades initiated by MAPK activation are essential in regulating cellular processes such as proliferation, differentiation, stress responses, and apoptosis (Cobb, 1999). Specifically, DON-mediated activation of MAPKs is known to initiate a ribotoxic stress response that ultimately leads to induction of apoptosis (reviewed in Pestka, 2008). Rapid regeneration of cells is essential for highly-proliferating tissues such as the liver, gut, and immune tissues and cells; however, the adverse effect of DON alters the generation of cells which can impede overall tissue function (Bondy and Pestka, 2000; Döll et al., 2003; Bony et al., 2006; Pinton and Oswald, 2014). Moreover, MAPK cascades also play a critical role in signal transduction in immune responses (Dong et al., 2002). When MAPK cascades are activated, transcription factors are altered to increase the expression of pro-inflammatory cytokines. In addition to apoptosis and protein synthesis inhibition, DON causes MAPK-mediated upregulation of both cytokines and chemokines that favour inflammatory pathways (Zhou et al., 2003). In practice, exogenous activation of MAPKs by DON translates into anorexia, reduced weight gain, altered nutritional efficiency, tissue injury and immunomodulation when exposure is prolonged (reviewed in Pestka, 2007).

1.2.2 Zearalenone (ZEA)

Although classified as non-steroidal estrogen, ZEA is structurally similar to 17β-estradiol and thus the adverse effects of ZEA are primarily attributed to its ability to mimic and compete with endogenous estrogens (Metzler et al., 2010). In terms of estrogenic capacity, ZEA enters the cytoplasm of cells via passive diffusion where it binds dormant, nuclear estrogen receptors and subsequently initiates shedding of heat shock proteins, acquisition of co-activators, and the formation of homo- and heterodimers. Activated dimers then bind estrogen response elements (ERE) in regulatory areas of DNA responsible for estrogen-responsive messenger RNA (mRNA) synthesis and subsequent production of gene products. Beyond genomic mechanisms, there is evidence that ZEA exerts estrogenic effects by binding both cytoplasmic estrogen receptors

(Tiemann et al., 2003) and membrane receptors (G-protein coupled receptors; He et al., 2018). Importantly, ZEA and its metabolites do not have equal affinity for estrogen receptors. Generally, ZEA is metabolized by dehydrogenases to either α-or β-zearalenol (ZEL). The affinity of β-ZEL for the estrogen receptor is approximately 2.5 times lower than ZEA while the affinity of α-ZEL is approximately 92 times higher (Fitzpatrick et al., 1989). The biotransformation to β-ZEL is therefore regarded as an inactivation pathway, whereas the biotransformation to α-ZEL is seen as bioactivation (Malejinekad et al., 2006).

1.3 Toxic effects of DON and ZEA with special reference to poultry

The recommended limit for DON inclusion in poultry diets is among the highest for production animals at 5 mg/kg (parts per million, ppm), while limits for other monogastrics, such as humans and swine, are restricted to 1 mg/kg (CFIA, 2015; European Commission 2006; FDA 2012). Poultry are widely considered to be resistant to DON, mainly owing to rapid clearance of DON and low bioavailability (<1%) at the tissue level (Prelusky et al., 1986). Moreover, large intestine fluids (colon and ceca) from laying hens have demonstrated the capacity to detoxify DON (Lun et al. 1988; He et al. 1992). Despite the observed tolerance of poultry to DON, there is evidence that poultry species respond adversely to feed at inclusion levels below 5 mg/kg (Awad et al., 2006; Awad et al., 2011; Awad et al., 2013; Antonissen et al., 2014).

There is limited data on the toxicity of ZEA to poultry reported to date. Early studies found that dietary exposure to ZEA up to 800 mg/kg had no effect on the performance, morphology, fertility, reproductive histology or hematology of poultry (Chi et al., 1980; Allen et al., 1981). Less information is available regarding the toxicokinetics of ZEA in poultry but according to Malejinekad et al. (2006), the less-estrogenic metabolite of ZEA, β-ZEL, is the primary product of ZEA metabolism in the chicken liver and poultry are regarded as ZEA-resistant. While regulatory guidelines exist for in-feed DON inclusion, major governing bodies do not currently impose dietary limits for ZEA in poultry feed (CFIA, 2015; European Commission 2006; FDA 2012) meaning that high levels of ZEA can occur in poultry diets alongside DON.

1.3.1 Oxidative stress

Oxidative stress arises when the concentration of reactive oxygen species (ROS) exceeds the antioxidant capacity of cells/tissues (Sies, 1991). Excess ROS then are able to elicit cell

damage by directly targeting critical macromolecules such as DNA, proteins, and lipids. If enough cell damage is incurred, ROS-dependent cell death can be stimulated through either necrotic or apoptotic pathways. Recently, oxidative stress pathways have been highlighted as an additional mechanism of DON toxicity with *in vitro* exposures to DON demonstrating DON-dependent ROS generation (reviewed in Mishra et al., 2014). Increased concentrations of ROS were found in immune (chicken splenic lymphocytes and isolated broiler lymphocytes) and non-immune (chicken embryo fibroblasts) cells following *in vitro* exposure to DON (Ren et al., 2015; Lautert et al., 2014; Li et al., 2014; respectively). An early study in mice reported that dietary exposure to DON increased hepatic lipid peroxides by 21% (Rizzo et al., 1994). In the studies of Lautert et al. (2014) and Li et al. (2014), exposure to DON and subsequent accumulation of ROS was accompanied by increase malondialdehyde (MDA) levels – the primary product of lipid peroxidation. This is supported by *in vivo* evidence that demonstrated a similar elevation of MDA in the jejunum (Awad et al., 2014), liver, and kidney (Borutova et al., 2008) of broiler chickens receiving DON-contaminated diets. Lipid peroxidation initiated by ROS causes damage to phospholipids and lipoproteins of the cell membrane and is a primary mechanism for ROS-mediated oxidative damage (Braca et al., 2002). Protection from ROS is mainly accomplished by the antioxidant enzyme glutathione (GSH) and its co-substrate glutathione peroxidase (GPx). Reduction in GSH (Li et al., 2014) and activity of GPx (Dragomir et al., 2007; Costa et al., 2009; Ren et al., 2015) have been observed *in vitro* in response to DON exposure. Taken together, evidence suggests that DON not only increases oxidative damage but also inhibits mechanisms that protect against oxidative stress, likely increasing the oxidative capacity of DON. Moreover, oxidative damage by DON can lead to cell death as evidenced by increased apoptosis in chicken splenic lymphocytes (Ren et al., 2015) and strong upregulation of pro-apoptotic genes following DON exposure (Benassi et al., 2012; Li et al., 2014; Ren et al., 2015). Comparable apoptotic effects have been seen *in vivo* where Taiwan country chickens, after a 16-week dietary exposure to DON, showed increased apoptotic cells and DNA damage within the spleen (Chen et al., 2017).

While estrogenicity is the most recognized mode of ZEA toxicity, not all adverse effects can be solely attributed to estrogenicity alone (Abid-Essefi et al., 2004). In fact, oxidative damage is a contributing pathway for ZEA toxicity (Hassen et al., 2007), although exact mechanisms for ZEA-induce oxidative stress are not yet established (Zinedine et al., 2007). Mitochondrial dysfunction was strongly implicated in ZEA toxicity where exposure of chicken

splenic lymphocytes to ZEA resulted in altered calcium homeostasis, intracellular acidification and eventual apoptosis (Wang et al., 2016). ZEA was also found to target mitochondria in human colon cells and this effect was linked to other observed effects of ZEA including increased lipid peroxidation and cell death and inhibition of protein and DNA synthesis (Kouadio et al., 2005). Numerous other *in vitro* studies have reported similar oxidative effects of ZEA including inhibited cell proliferation and increases in ROS production, apoptotic cells, heat shock protein expression and MDA formation (Abbid-Essefi et al., 2004; El Golli-Bennour et al., 2008; Bouaziz et al., 2008; Abbid-Essefi et al., 2009; Lee et al., 2013). Animal studies using dietary exposures to ZEA support the *in vitro* findings. Pregnant rats fed ZEA for seven days exhibited decreased activity of antioxidant enzymes and increased MDA content in both serum and liver at all levels tested (Zhou et al., 2015). These same responses, along with increased liver weight, were observed in gilts following dietary exposure to low levels of ZEA (1.1 to 3.2 mg/kg) for 18 days (Jiang et al., 2011). Only one dietary exposure to ZEA in poultry evaluated biochemical indicators related to oxidative stress. In this study, laying hens exposed to 7.9 mg/kg ZEA for 4 weeks demonstrated an oxidative stress response through elevated activity of GPx in the liver and kidney and increased activity of γ-glutamyltransferase in the plasma although no clinical toxicosis was observed (Gresakova et al., 2012). While the exact mechanisms by which ZEA induces oxidative stress are unknown, it is clear that, in the absence of overt mycotoxicosis, ZEA may still produce oxidative damage that sub-clinically affects the overall health of poultry.

1.3.2 Immunomodulation

In poultry, as well as other species, DON exhibits both immunostimulatory and immunosuppressive effects depending on the concentration of DON administered, the duration of exposure, and the sensitivity of the exposed species (Pestka, 2008). Generally, low concentrations of DON (< 5 ppm) or acute exposures stimulate the immune system whereas high concentrations or chronic exposure suppress it (Pestka, 2003; Swamy et al., 2004). Although immune modulation by DON is not completely understood, it is likely due to DON-mediated activation of MAPK cascades or DON-induced oxidative damage as both pathways can affect immune tissues and cells. The spleen, specifically, appears to be a target for DON *in vivo*. Increased germinal centers were identified in spleens of turkey poultry following dietary exposure to 3.9 mg DON/kg feed for up to three weeks (Girish et al., 2010). Taiwan country chickens fed 5 mg DON/kg feed for 16 weeks had heavier spleens which, upon histological analysis, was attributed to an increased

number of splenic germinal centers as compared to groups receiving lower concentrations of DON (Chen et al., 2017). Similarly, broiler chickens fed a low-level DON diet (1.68 mg DON/kg feed) displayed increased spleen weight after four weeks of exposure (Yunus et al., 2012). In this study, splenic changes induced by DON were accompanied by altered antibody titers in response to common poultry vaccines (infectious bronchitis virus and Newcastle's disease virus). *In vivo* studies have reported an ability of DON to suppress antibody responses to vaccines for both infectious bronchitis virus (Ghareeb et al., 2012) and Newcastle's disease virus (Dänicke et al., 2003) in poultry. In a recent study, broilers fed a diet naturally contaminated with a complement of *Fusarium* toxins, including DON, had reduced IgA, relative spleen weight, and antibody titers against NDV (Li et al., 2012). The same study found that DON also modulated the expression of various pro- and anti-inflammatory cytokines in the spleen. Within cells, DON modifies intracellular transcription factors via action at the ribosome, potentially altering expression of chemokines and cytokines and the immune and inflammatory pathways they regulate (Zhou et al., 2003; Pestka, 2007). This function of DON may, in part, explain leukocytic responses to DON. Populations of duodenal lymphocytes and circulating monocytes were reduced and the metabolic burst of heterophils was increased in broilers fed 3 mg DON/kg feed for two weeks (Revajova et al., 2013). In broiler chicks fed 3 mg DON/kg feed for four weeks, a similar increase in circulating heterophils was noted (Levkut et al., 2009). Feeding of naturally DON-contaminated diets over a longer period (eight weeks) was found to decrease B- and T-cell counts in broilers (Swamy et al., 2004). In addition to direct effects on immune tissues and cells, Antonissen et al. (2014) found that intake of DON-contaminated feed (3 – 4 mg DON/kg feed) and subsequent damage to intestinal mucosa was a pre-disposing factor for the development of bacterial disease in broilers. Thus, low-level DON contamination, while not directly responsible for disease, can reduce efficiency of production and increase susceptibility to disease.

While immunotoxic mechanisms of ZEA have not been fully elucidated, it is likely that ZEA toxicity to the immune system can be conferred in multiple ways. Firstly, ZEA is structurally similar to estrogen and the immune system is sensitive to estrogenic compounds due to the fact that many immune cells possess estrogen receptors. Estrogenic compounds, like ZEA, can be immunostimulatory or immunosuppressive depending on the dose and timing of exposure (Igarashi et al., 2001). Secondly, ZEA also possesses an oxidative capacity (reviewed above) and highly-proliferating cells, such as immune cells and tissues, are susceptible to oxidative stress (Bony et al., 2006; Pinton and Oswald, 2014). Indeed, *in vitro* treatment with ZEA increased

markers of lipid peroxidation and activity of acetylcholinesterase in isolated broiler lymphocytes (Lautert et al., 2014). Exposure to ZEA has also been shown to alter mRNA expression of cytokines in chicken splenic lymphocytes *in vitro* (Wang et al., 2012) as well as in pig spleens following 18-day dietary exposure (Pistol et al., 2015). Immune responses to ZEA *in vivo* appear to be immunostimulatory. Specifically, lymphoid infiltration of the liver has been observed in both mice (Abbès et al., 2006a) and swine (Jiang et al., 2010) consuming ZEA contaminated diets. Dietary exposure of goats to low-level ZEA-contaminated diets (2.4 mg ZEA/kg body weight) also resulted in hepatic lymphoid infiltration, which was associated with increased expression of estrogen receptors in the liver (Dong et al., 2010).

1.3.3 Lipid utilization and metabolism

The liver is a highly metabolic organ and is partially responsible for the detoxification of ingested mycotoxins, including DON and ZEA. However, to maintain metabolic function, the liver requires constant cell turnover and, as one of the first organs to encounter unmetabolized DON and ZEA, the liver is also susceptible to their toxic effects. The liver plays an important role in lipid metabolism, storage, and distribution, and all of these processes can be disrupted by DON or ZEA to affect lipid homeostasis. Serum concentrations of total cholesterol and triglycerides were elevated in mice fed very low doses of DON (45 μg/kg body weight/day) for seven days (Kouadio et al., 2013). Apart from this study, DON-associated impacts on lipid metabolism appear to manifest in the liver. Dietary exposure to DON significantly increased total lipids in livers of carp (Pietsch et al., 2014; 953 μg DON/kg feed) and total cholesterol and triglycerides in livers of laying hens (Farnworth et al., 1983; 0.35 mg DON/kg feed). A single dose of DON (2 μg DON/g egg weight) administered *in ovo* to chicken embryos (embryonic day 12, ED12) resulted in accumulation of fatty droplets in the liver (Moon et al., 2007).

Altered lipid profiles have been observed following *in vivo* exposure to ZEA. Blood triglycerides and hepatic lipids were increased in mice exposed to 200 μg DON/day over 14 days (Nogowski, 1996). In this case, increased blood triglycerides were attributed to the actions of insulin; however, insulin does not promote lipid accumulation, so this effect was hypothesized to be due to anabolic activity of ZEA. Pigs receiving 1.3 mg DON/kg feed for 24 days demonstrated elevated cholesterol, high-density lipoprotein, and reduced triglycerides in serum (Jiang et al., 2010). While this study offered no direct mechanism of action, the potential estrogenic metabolic effect of ZEA was suggested. A single dose of ZEA (40 mg ZEA/kg body

weight) significantly reduced levels of total cholesterol, triglycerides and high- and low-density lipoproteins in the serum of mice (Abbès et al., 2006b). Unlike previous studies, Abbès et al., (2006b) postulated that effects of ZEA on the lipid profile resulted from ZEA-induced liver stress rather than estrogenic effects of ZEA. Overall, the effects of ZEA on lipid metabolism has not been investigated in poultry but based on results in other animals ZEA could potentially alter lipid metabolism via estrogenic or oxidative pathways.

1.3.4 Combined toxicity of DON and ZEA

Together, DON and ZEA comprise one of the most frequently detected combinations of mycotoxins (Smith et al., 2016), yet relatively little is known about their interactive toxicity. Studies conducted with cell lines indicate that DON and ZEA behave both additively and synergistically but have limited estrogenic effects. Swine jejunal epithelial cells exposed to a combination of DON and ZEA were found to have decreased cell viability, despite the fact that the individual doses of each mycotoxin were non-cytotoxic (Wan et al., 2013). In human colon cells, DON and ZEA demonstrated a nearly-additive reduction in cell viability (Kouadio et al., 2007). Lymphocyte proliferation was also reduced in an additive and dose-dependent manner in human and rat cell lines (Atkinson and Miller, 1984). In a study by Sun et al., (2015), synergistic interactions were reported between aflatoxin B_1 (AFB) and DON, as well as AFB and ZEA in rat liver cells and these combinations decreased cell viability through increased production of ROS and induction of apoptosis, as indicated by up-regulation of caspases and down-regulation of an anti-apoptotic gene. The combined toxicity of DON and ZEA was not investigated in the aforementioned study; however, potential interaction between DON and ZEA is suggested by similar interactions with AFB. Regarding estrogenic effects, Pizzo et al. (2016) found no interactive effects of the two toxins on cell proliferation or steroidogenesis in bovine ovarian cells.

There are several *in vivo* studies that suggest that DON and ZEA together can have adverse effects on the immune system of animals. In a study by Pestka et al. (1987), exposure to DON and ZEA together reduced resistance to infectious bacteria, *Listeria monocytogenes*, in mice to a greater extent than DON alone, while Liang et al. (2015) found that mice receiving intraperitoneal injections of the combination displayed sub-additive nephrotoxic effects. In the latter study, the exposure was found to increase the rate of apoptosis and increase indicators of renal oxidative stress (serum creatine and urea). Conversely, when mice received the

combination of DON and ZEA orally, the effect of DON and ZEA was determined to be antagonistic regarding lipid peroxidation and anti-oxidant capacity in the liver and synergistic regarding hepatic mRNA expression of pro-apoptotic genes (Sun et al., 2014). An early report in weanling mice found no synergistic or antagonistic effect of DON + ZEA feeding on organ weights and immune function (Forsell et al., 1986). However, this study did not indicate whether additive effects were present and endpoints to evaluate immune function were limited. Among livestock, swine are regarded as one of the most sensitive species to mycotoxins. Pigs fed naturally contaminated diets displayed decreased growth performance, increased oxidative DNA damage and increased liver hydropic degeneration in response to a combined DON and ZEA exposure compared to a non-exposed group; however, no contrast between individual mycotoxins and the combination was offered (Weaver et al., 2014). Young pigs fed low-levels of DON (0-3.0 mg/kg) and varying concentrations of 15-ADON and ZEA exhibited metabolic effects including, decreased skin temperature, feed intake, and thyroid size in addition to a delayed immune response (Rotter et al., 1994). Even at very low levels (μg/kg body weight range), pigs fed the combination of DON and ZEA showed increased lymphocytes, plasma cells, and macrophages in the lamina propria of the gastrointestinal tract – however these immune effects were not considered to be additive or synergistic (Lewczuk et al., 2016). In broiler chickens, intake of both high and low levels of DON and ZEA resulted in decreased peripheral lymphocytic and phagocytic cell populations; however, again, this study did not contrast individual effects of DON or ZEA with their combinatorial effects (Levkut et al., 2011).

1.4 Challenges evaluating mycotoxin toxicity

1.4.1 Inter- and intra-species sensitivity

Not all species are equally susceptible to the effects of mycotoxins, making it difficult to extrapolate conclusions based on toxicity data from one species to another. For example, the widely documented sensitivity of animals to DON from most to least sensitive is ranked as follows: pigs > mice > rats > poultry ≈ ruminants (Prelusky et al., 1994). Differences in sensitivity are generally attributed to the varying types of gastrointestinal systems (i.e. monogastric vs. ruminants, hindgut vs. foregut fermenters) which contribute to varying mycotoxin toxicokinetics (i.e. absorption, distribution, metabolism and elimination). Depending on the toxicokinetic mechanisms employed by a species, mycotoxins can either be

biotransformed to bioactive (more toxic) or inactive (less toxic) metabolites, which confer increased or decreased sensitivity, respectively.

In poultry, there is wide variability in species-specific sensitivity to mycotoxins. For example, turkeys have shown greater sensitivity to DON as compared to chickens in part due to slower excretion of DON and its metabolites (Schwartz-Zimmerman et al., 2015) and ZEA (Devreese et al., 2015) when compared to chickens. Similarly, greater susceptibility of turkeys to ZEA is attributed to the predominant hepatic metabolism of ZEA towards the production of a bioactivated metabolite (α-ZEL) when compared to metabolic capacity of chickens (Devreese et al., 2015). Even within a single poultry species, comparing responses to mycotoxins can be complex. Broiler and layer chickens, despite their genetic proximity, have been consistently bred for different purposes and thus, differ greatly in their physiology and their metabolic capacity (Buzala et al., 2015). Huff et al. (1986) concluded that the rapid growth of broilers made them more sensitive to growth rate inhibition induced by DON, as compared to layers. Differences in susceptibility to DON is also reported among broiler breeds. Earlier studies using the Hubbard strain found that a diet containing greater than 15 mg DON/kg feed was required to affect growth performance (Huff et al., 1986; Kubena et al. 1988, 1989). However, more recent studies in Lohmann (Dänicke et al., 2003) and Ross (Swamy et al., 2004; Yunus et al., 2012; Ghareeb et al., 2014) strains have found reduced performance at DON levels ranging from 7 to 12 mg DON/kg feed and this difference was again attributed to faster growth. Yunus et al. (2011) postulated that improved growth rate and feed efficiency in modern broilers decreases their threshold for mycotoxin-induced effects on growth performance. While less information is available with regard to chicken sensitivity to ZEA, it is likely that these differences, as well as the genetic and microbial diversity that may occur in one flock, have potential to contribute to variability in toxicity and make it difficult to compare findings across studies or apply them to a production setting.

1.4.2 Experimental design and modelling

The type of experimental model or certain factors of experimental design can affect the outcome of mycotoxin toxicity studies. *In vitro* methods are useful for evaluating effects on specific cells or tissues, but it is difficult to accurately account for systemic influences, like toxicokinetics, that can increase or decrease the potency of mycotoxins. Moreover, these studies are generally only conducted over short periods of time (i.e. 24 to 96-hour exposures) and do not

reflect a chronic exposure. Conversely, *in vivo* methods present another unique set of challenges. When formulating diets for *in vivo* mycotoxin exposures, one must focus not only on the mycotoxin content of the diet but also on ensuring the contaminated diet is ingested, which can be difficult as many mycotoxins are known to impact feed intake (Eriksen and Petterson, 2004). Force-feeding is the only way to eliminate this variation in feed intake *in vivo*; however, this practice presents serious welfare concerns. In addition, duration and level of exposure can influence the severity of effects observed with dietary mycotoxin exposure. In terms of immunity, responses to low concentrations of DON or acute exposures are often associated with immune stimulation, while those induced by high concentrations of DON or chronic exposures are more in line with immune suppression (reviewed in Pestka, 2008). In a study by Wang and Hogan (2018), broiler chickens were more sensitive to the adverse effects of DON on growth performance in the latter part of the production cycle (grower phase), demonstrating that timing of exposure with regard to developmental stage can also influence experimental outcomes.

The use of either artificially or naturally mycotoxin-contaminated diets in animal trials can also influence the degree of toxicity. Because mycotoxins rarely exist independently of one another within feeds (Schatzmayr and Streit, 2013; Smith et al., 2016; Biomin, 2017), there is risk of mycotoxin synergism or additivity in feeds that are formulated with naturally-contaminated grain. While this situation is more reflective of feed used in commercial production systems, interactive toxicity can lead to unintentional over- or under-estimation of mycotoxin toxicity when the diet is referred to as mono-contaminated (i.e. referred to as "DON-contaminated" when smaller quantities of other mycotoxins are also present).

1.4.3 Analyzing interactive toxicity

As previously stated, animal feed is particularly vulnerable to contamination by multiple mycotoxins and studying their interactive toxicity provides more relevant information to the livestock industry. Interactions between mycotoxins are inferred when a mixture of mycotoxins produces a biological response that is greater (synergistic) or lower (antagonistic) than expected based on their individual toxicities. Interactions can also be defined as potentiative when one or both of the mycotoxins in a binary mixture does not induce an effect whereas the combination induces a significant response. When the effect induced by a mycotoxin combination is equal to the sum of their individual effects, this is considered additivity (which is a non-interaction effect).

Currently, the three main approaches to analyzing interactive toxicity are 1) the arithmetic definition of additivity, 2) factorial designs, and 3) the theoretical biology-based methods (Alassane-Kpembi et al., 2017). In their review of combinatorial mycotoxin research, Alassane-Kpembi et al. (2017) determined that most studies relied on arithmetic definitions of additivity to determine interactive toxicity of mycotoxins. With this approach, combined effects of mycotoxins are arithmetically contrasted to the individual (expected) effects and values that are not significantly different, or that are above or below the expected values, are interpreted as additive, synergistic, or antagonistic, respectively. This is not considered a robust method as it does not provide a reasonable reference point for interactivity (Boedeker and Backhaus, 2010; Alassane-Kpembi et al., 2017). In contrast, theoretical biology-based methods (Bliss' independent criterion, Loewe's additivity model, the Chou-Talay method, and isobolograms) allow researchers to determine the presence of an interaction, the type of interaction and, optionally, the interaction magnitude. These models, while considered very biologically plausible, require researchers to determine a median-effect dose (*i.e.* IC_{50} or EC_{50}) for measured endpoints. *In vivo*, median-effect doses would need to be determined for each measured parameter as they are likely to differ greatly across endpoints in a whole animal system. Therefore, this approach is not feasible for *in vivo* experimentation. The most robust method for determining *in vivo* mycotoxin interactions, thus far, is the factorial design method as it allows researchers to test a true, statistical departure from additivity. Unfortunately, the nature of interaction with regard to additivity, synergism or antagonism cannot be determined with this approach and has to be inferred indirectly (Bhat and Ahangar, 2007). A factorial design applied to animal feeding studies can also be costly, resource intensive and use large numbers of animals as each mycotoxin in the mixture must also be tested individually.

1.5 The chicken embryo as an alternative animal model for mycotoxin research

Discerning the effects of nutritional toxins can be a complex process and mycotoxins are no exception. When studying mycotoxins and their interactions *in vivo*, the process is further complicated by the inherent variability surrounding whole animal (*in vivo*) models. Considering the limitations of both *in vitro* and *in vivo* research (described in the sections above), alternative models for characterizing mycotoxin toxicity should be explored. The developing chicken embryo, or *in ovo* model, maintains the benefits of both *in vitro* and *in vivo* modelling by providing a whole animal system within the isolated environment of the egg. Compared to *in*

vivo models, the nutritional self-sufficiency of the chicken embryo and direct administration of test compounds *in ovo* greatly limits the sources of variation previously discussed. Therefore, this model, being a whole organism, could achieve similar objectives to the *in vivo* model but its use would greatly improve the efficiency of experimentation - effectively reducing the number of individual animals used. The chicken *in ovo* model is also accessible (locally or commercially), easy to handle, and typically inexpensive, allowing researchers from a diverse set of backgrounds and skill levels to utilize them (Bjørnstad et al., 2015; Scanes and McNabb, 2003; Henshel et al., 2002). Moreover, the short incubation time of the chicken egg (~21 days) means it can function as a high-throughput screening model (Bjørnstad et al., 2015). Taken together, these characteristics allude to a favorable alternative for effectively assessing the toxicity of individual mycotoxins and mycotoxin mixtures on major organ systems.

The chicken embryo is an extensively studied model and the process of its development is well-documented; thus, information regarding the anatomical, physiological, biochemical and endocrine development of many of genetic sources/breeds of chick embryos is copious (Scanes and McNabb, 2003). The value of *in ovo* testing of environmental contaminants has already been recognized. Toxic effects of a diverse range of pollutants, including perfluorooctanesulfonate (Peden-Adams et al., 2009), polychlorinated biphenyls (Carlson and Duby, 1973), polyhalogenated aromatic hydrocarbons (DeWitt et al., 2005), bisphenol-S (Crump et al., 2016), methylmercury (Heinz et al., 2006), thiurams (Korhonen et al., 1982) and bendiocarbs (Petrovova et al., 2009), have been successfully evaluated by using chicken embryos as wild-life surrogates. Additionally, established *in ovo* assays such as the chicken embryo screening test (CHEST; Jelinek et al., 1994) and the early embryo assay (EEA; Henshel et al., 1993) can be used to study embryotoxicity of pharmacologic compounds. These assays focus on insult during early embryonic development; thus, similar assays have been used to study maternally-deposited mycotoxins (aflatoxin B1, ochratoxin, citrinin) that appear in the egg environment during this time. Such studies demonstrate that the individual (Oznurlu et al., 2012; Saleemi et al., 2015; Monson et al., 2016) and combined (Veselá et al., 1983; Edrington et al., 1995) toxicity of mycotoxins can be evaluated across morphological, histological, biochemical and molecular endpoints in the early-stage chicken embryo. Therefore, individual and interactive effects on these endpoints can likely be evaluated following a late-term exposure (embryonic day 14 – hatch) as well. By embryonic day 14, the chicken embryo is fully formed, and the remainder of incubation is dedicated to growth and refinement (Figure 1.2). Mycotoxin exposure aimed at this

period would limit potential teratogenic effects and would provide a more suitable model for predicting *in vivo* responses to non-maternally deposited mycotoxins (like DON and ZEA). Moreover, embryonic development during this time is defined by production of distinct immune cells as well as the rapid growth and increased functionality of the gastrointestinal tract (Romanoff, 1960; Romanoff and Romanoff, 1972; Macalintal, 2012). Similarly, the development of starter phase chickens (post-hatch day 1 – 21) is characterized by rapid growth of the small intestine and diversification of the immune system. It is likely, then, that responses of chicken embryos following late-term, *in ovo* exposure to mycotoxins could be more easily extrapolated to production animals than *in vitro* responses to the same mycotoxins.

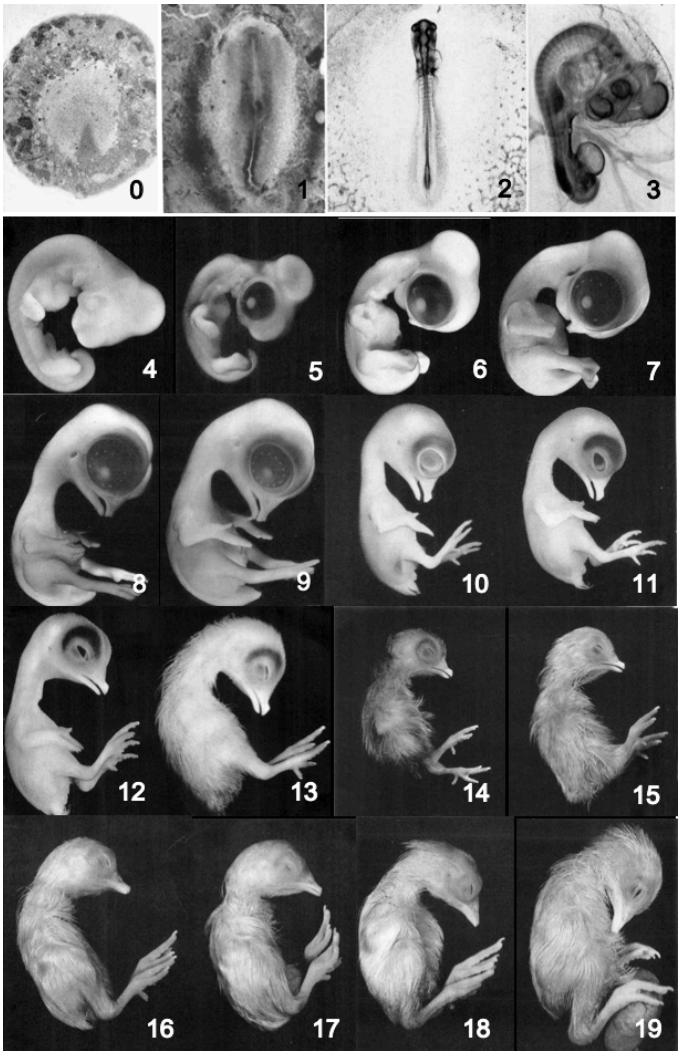

Figure 1.2 Embryonic development of the chicken embryo throughout incubation. Number labels for each image indicate day of incubation or embryonic day (ED). Adapted from Hamburger and Hamilton (1951).

The chicken egg is a versatile model for toxicity testing that allows multiple routes of toxin exposure to be mimicked *in ovo* by administering test compounds to specific egg structures. For example, toxins applied to the shell mimic exposure to environmental contaminants (e.g. pesticides) (DeWitt et al., 2005) and vaporized toxins within an incubation chamber, post-pip, replicate the route of inhalation (Bjørnstad et al., 2015). Toxins can also be injected into internal egg components. Injection into the highly vascularized yolk sac allows researchers to imitate systemic or maternal exposure (Bjørnstad et al., 2015). In the case of mycotoxins, animals are exposed through the diet, thus a successful test model for combined mycotoxin toxicity must be able to mimic exposure via ingestion. For replicating the route of exposure for ingested contaminants, the toxins can be injected into the amniotic fluid of the egg during the last third of the incubation period (beginning embryonic day 14). Immediately prior to this period, the albumen sac ruptures and albumen begins to move freely into the amniotic sac (Oegema and Jourdian, 1974). This mixture of amnion and albumen is then orally consumed by embryo (Oegema and Jourdian, 1974; Henshel et al., 2002), absorbed within the gastrointestinal (Sugimoto et al., 1999; Moran Jr., 2007) and is metabolized as feed (Sugimoto et al., 1999; Bjørnstad et al., 2015). A small portion of the albumen also moves into the yolk sac, evading oral consumption (Moran Jr., 2007). For brevity, the term amniotic fluid will hereafter refer to the mixture of amniotic fluid and albumen mixture.

In conclusion, the *in ovo* model is a promising intermediate between *in vivo* and *in vitro* approaches for evaluating the toxicity of singular and co-occurring mycotoxins. *In ovo* methodology can effectively reduce the number of animals required for individual and combinatorial mycotoxin exposures as compared to *in vivo* modelling while still retaining some precision of cell model exposures. This model is accessible, versatile, and due to its background of use in toxicity testing, the knowledge regarding the development of this model is widely available. While traditional *in ovo* assays have focused on toxin exposure at early stages of embryonic development, an amniotic fluid exposure targeted at development during late incubation would more closely resemble dietary exposure in the starter-phase chicken and observed physiological responses could more easily be extrapolated to practical application. Overall, the *in ovo* model could be used to rapidly-screen mycotoxins in order to predict whole animal responses and prioritize single mycotoxins, or mycotoxin mixtures, for future investigation *in vivo*. In the future, this model could also be used to test mitigation techniques for the effects of mycotoxins and elaborate on the potential detrimental effects of emerging

mycotoxins, mycotoxin metabolites and masked mycotoxins (conjugates of well-known mycotoxins).

1.6 Objectives and Hypotheses

The overall objective of this thesis research is to determine whether an *in ovo* approach for conducting exposures to *Fusarium* mycotoxins could be used as a predictive tool for assessing the toxicity of *Fusarium* mycotoxins alone and in combination. The overall hypothesis of this thesis research is that responses of the late-term chicken embryo to single doses of *Fusarium* mycotoxins, alone or in combination, are similar to those reported in whole animal feeding trials with poultry. In order to test this hypothesis, a series of specific objectives are identified:

1. To determine the impacts of a single, *in ovo* administration of DON to the late-term chicken embryo in terms of embryotoxicity, growth and development, pathological changes to tissue, and biochemical/molecular indicators of oxidative stress and immune response.

2. To determine individual and interactive effects of a single, *in ovo* administration of DON and/or ZEA to the late-term chicken embryo in terms of embryotoxicity, growth and development, pathological changes to tissue, and biochemical indicator of oxidative stress.

3. To compare outcomes of *in ovo* exposures to previous *in vitro* and *in vivo* research in poultry reporting individual effects of DON and the effects of DON when in combination with ZEA.

CHAPTER 2

PATHOLOGICAL AND PHYSIOLOGICAL RESPONSES OF CHICK EMBRYOS INDUCED BY *IN OVO* EXPOSURE TO THE *FUSARIUM* MYCOTOXIN DEOXYNIVALENOL

PREFACE

In this chapter, we examined DON toxicity *in ovo* by evaluating morphological, histopathological, functional and gene expression parameters. A single injection of purified DON was administered to late-term chicken embryos at five doses ranging from 0.0 – 5.0 μg DON/g egg weight. The chicken embryo demonstrated a clear response to DON with effects on immunity and oxidative stress that were comparable to previous *in vivo* and *in vitro* findings. Our results indicate that *in ovo* mycotoxin exposures can, to a certain extent, mimic dietary mycotoxin exposure and predict manifestations of mycotoxin toxicity *in vivo*.

Author contributions:

Émilie Viczko (University of Saskatchewan) helped design the experiment, conducted the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Susantha Gomis (University of Saskatchewan) provided histopathological expertise, evaluated the slides and shared his interpretation.

Dr. Lynn Weber (University of Saskatchewan) and her research assistant, Jennifer Briens, provided initial guidance and expertise for conducting the lipid peroxidation assay.

Dr. Natacha Hogan (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and obtained funding for the research.

2.1 Introduction

Infection of cereal grains by *Fusarium* fungi is a major cause of plant diseases in crops worldwide. Apart from losses in grain yield and reduction in seed quality, the major concern with fungal infection is the associated contamination of food and feed ingredients by toxic fungal secondary metabolites, known as mycotoxins. *Fusarium* mycotoxins elicit acute and chronic toxicity and as such, pose a significant risk to human and animal health, resulting in substantial economic losses within the animal production sector (Hussein et al., 2001). Mycotoxins present in raw food and feed commodities cannot be eliminated through typical processing methods and often end up in finished food products and livestock diets (Sugita- Konishi et al., 2006; Bullerman and Bianchi, 2007; Grenier and Oswald, 2011). The fusario-mycotoxin deoxynivalenol (DON), produced mainly by *F. culmorum* and *F. graminearum*, is acknowledged as the most abundant mycotoxin globally (Rodrigues and Naehrer, 2012). In a global survey of feed and raw feed materials, DON was found to be the most prevalent mycotoxin, appearing in 55% of all samples tested (Streit et al., 2013).

Poultry are considered to be somewhat resistant to DON, in part due to rapid clearance and low bioavailability of DON at the tissue level (Prelusky et al., 1986). Additionally, metabolism of DON to non-toxic metabolites has been linked to anaerobic fermentation. Indeed, DON incubated with large intestine fluids (colon and ceca) from laying hens is largely detoxified or biotransformed (Lun et al. 1988; He et al. 1992). The recommended limit for DON inclusion in poultry diets is among the highest for production animals at 5 ppm, while limits for other monogastrics, such as humans and swine, are restricted to 1 ppm (CFIA, 2015; European Commission 2006; FDA 2012). Despite the observed tolerance of poultry to DON, there is evidence that poultry species respond adversely to feed at inclusion levels below 5 ppm (Awad et al., 2006; 2011; 2013; Antonissen et al., 2014). This may be a result of interactions between co-occurring mycotoxins, the presence of undetectable toxic metabolites or masked mycotoxins, or be due to subclinical manifestations of DON toxicity.

The toxicity of DON is attributed to its potent inhibition of protein, RNA and DNA synthesis (Rotter et al., 1996). At the ribosome, DON is able to bind the 60S unit and initiate an MAPK cascade, ultimately inducing apoptosis as a part of the ribotoxic stress response (reviewed in Pestka, 2007). Thus, tissues that require a high level of protein turnover, such as the gut, immune tissues and the liver, are particularly susceptible to DON toxicity (Bondy and Pestka, 2000; Döll et al., 2003; Pinton and Oswald, 2014) and are among the first tissues to

encounter any unmetabolized, undetoxified DON which also increases their exposure (Smith et al. 2016). Organ-specific susceptibility to DON has been reflected *in vivo* where broiler chickens fed a low-level DON diet (1.68 mg/kg) exhibited increased liver and spleen weight during week two and four of exposure, respectively (Yunus et al., 2012). These changes were accompanied by a concurrent elevation in antibody titers to common poultry vaccines demonstrating immune, as well as organ, sensitivity to DON. Immunomodulation by low-level DON exposure was demonstrated in broilers where duodenal lymphocytes and circulating monocytes were reduced and the metabolic burst of heterophils was increased following a two-week feeding of 3.0 mg/kg DON-contaminated diet (Revajova et al., 2013). Beyond direct immune effects, intake of DON-contaminated feed and subsequent damage to intestinal mucosa was also shown to be a pre-disposing factor for the development of bacterial disease in broilers (Antonissen et al., 2014). Thus, low-level DON contamination, while not directly responsible for disease, can reduce efficiency of production and increase susceptibility to disease. DON is also able to alter intracellular transcription factors through the ribosomal pathway and, in turn, modify expression of chemokines and cytokines and the subsequent immune and inflammatory pathways they regulate (reviewed in Pestka, 2007). Reduced spleen weight, IgA levels and splenic mRNA expression of cytokines were observed in broilers fed a diet naturally contaminated with a complement of *Fusarium* toxins, including DON (Li et al., 2012). Increased concentrations of reactive oxygen species (ROS) and apoptotic proteins were also observed in chicken splenic lymphocytes exposed to DON *in vitro* (Ren et al., 2015).

While contamination of feeds with low and moderate levels of DON is a significant health concern for livestock production, most experimental *in vivo* studies examining DON toxicity in poultry show highly variable effects of DON on growth performance, gastrointestinal health and immune function (Awad et al., 2011; Yunus et al., 2012; Awad et al., 2013; Ghareeb et al., 2015). Differences in species, breed, developmental stage, feed composition, mycotoxin profile, exposure timing and duration may all contribute to variability across poultry studies. Many mycotoxins also influence feed intake, which makes consistent exposure difficult to control even within a single feeding trial. Thus, typical *in vivo* endpoints are subject to the inherent variability when using a whole animal model and may not be sensitive indicators of DON toxicity. Interpretation of results can be further confounded when considering potential interactions among co-occurring mycotoxins in naturally contaminated diets. Data obtained through *in vitro* modelling is typically used to predict interactions of mycotoxins (Ruiz et al.,

2011; Benassi et al., 2014; Clarke et al., 2014; Alassane-Kpembi et al., 2017), but *in vitro* responses do not completely agree with those observed *in vivo* (Tiemann and Dänicke, 2007). While they can provide useful information on mechanisms of action and have less variability in measured endpoints when compared to *in vivo* models, *in vitro* models are also void of potential systemic interactions that may, through microbial or enzymatic action, influence the toxicity of a mycotoxin and thus, do not completely reflect the complexity of a whole animal response.

The chicken *in ovo* model is an under-utilized tool in the study of mycotoxin toxicity to poultry. The chicken embryo has been widely, and successfully, used as a non-animal alternative to evaluate the toxicity of environmental pollutants, including perfluorooctanesulfonate (Peden-Adams et al., 2009), polychlorinated biphenyls (Carlson and Duby, 1973), polyhalogenated aromatic hydrocarbons (DeWitt et al., 2005), bisphenol-S (Crump et al., 2016), methylmercury (Heinz et al., 2006), thiurams (Korhonen et al., 1982) and bendiocarbs (Petrovova et al., 2009). Early embryo bioassays have been used to study mycotoxins that are maternally deposited in the egg (e.g. aflatoxin, ochratoxin and citrinin) and the potential toxicity they confer to the embryo during development. Adverse effects of these mycotoxins (alone or in combination) on developing embryos and hatching chicks are evaluated through occurrence of embryonic malformations, hatch parameters, morphology and histopathology of target organs, biochemical and molecular markers, and post-hatch growth (Edrington et al. 1995; Veselá et al. 1983; Oznurlu et al. 2012; Saleemi et al. 2015; Monson et al. 2016). Mid-term (embryonic day 11) *in ovo* exposure was used to develop a toxic profile for the fusario-toxin, butenolide (Wang et al., 2008; 2009). Although carry-over of DON from feed to the fertilized egg is negligible (Valenta and Dänicke, 2005), *in ovo* administration of DON during late term embryonic development could be used as a screening tool to test the combinatorial effects of mycotoxins and potential mitigation strategies. Embryonic development during this time is defined by production of immune cells and the rapid growth and increased functionality of the gastrointestinal tract (Romanoff, 1960; Romanoff and Romanoff, 1972; Macalintal, 2012), organ systems that are particularly sensitive to DON toxicity in growing birds. In fact, the development of chickens during the starter phase (post-hatch day 1 – 21) is similarly characterized by rapid growth of the gut and diversification of the immune system. Thus, results derived from DON exposure to late-term chicken embryos could serve as an intermediate between *in vitro* and *in vivo* models to better inform whole animal studies.

The aim of the present study was to gain a comprehensive understanding of DON toxicity *in ovo* and evaluate the validity of this model as an intermediate by comparing responses of the chick embryo to DON effects *in vivo* in previous poultry studies. To this end, analyses included evaluation of apical (hatch parameters, organ morphology), structural (liver and spleen histology), and functional (thiobarbituric acid reactive substances assay; TBARS assay) endpoints. Furthermore, genes related to oxidative stress and immunity were evaluated in the liver and spleen, respectively, to determine potential mechanisms of DON toxicity.

2.2 Materials and Methods

2.2.1 Eggs and treatment procedure

Two-hundred thirty fertilized Lohmann LSL x Bovan (female x male) eggs were obtained from the University of Saskatchewan layer flock and housed at the Poultry Research Centre at the University of Saskatchewan. All eggs were weighed prior to set (start of incubation). Set (ED1) began within 24 hours of lay for all eggs. The incubator was maintained at 37.5 ºC and 60% humidity from ED1 to ED14. At ED14, eggs were removed from the incubator and candled to identify unfertilized eggs or early dead embryos. Eggs containing live embryos were utilized for *in ovo* exposure via amniotic fluid injection.

Purified DON (CAS # 51481-10-8; purity $\geq$ 98%) and dimethyl sulfoxide (DMSO; CAS # 67-68-5) were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Solutions of purified DON were prepared at concentrations of 0.04, 0.2, 1.0 and 5.0 µg DON/µL in 10% DMSO. Each viable embryo was randomly allocated to one of six treatment groups (n=30): four DON treatment groups, an untreated control group (CON) and a vehicle-injected control group (DMSO) administered only the vehicle solution of 10% DMSO in purified water. On ED14, each solution was administered at a volume of 1 µL/g of egg weight (measured on ED1) such that eggs received 0.0, 0.04, 0.2, 1.0 or 5.0 µg DON/g egg weight. These groups will hereby be referred to as DMSO, DON 0.04, DON 0.2, DON 1.0, and DON 5.0, respectively. Immediately preceding injection, egg surfaces were disinfected with a 70% ethanol solution. Using a diamond-tipped Dremel tool, a hole was drilled in the shell at the air cell-end of each egg. The appropriate solution was drawn using a 200-µL pipette fitted with a sterile gel-loading tip (VWR), inserted into the hole at an ~45º angle and released into the amniotic fluid of the egg. Following injection, holes were sealed with silicone rubber (GE Silicon I, all-purpose, clear).

Pipette tips were changed between each injected egg. Following treatment, eggs were returned to the incubator until ED18 at which time they were transferred to a second incubator (37.5 ºC, 70% humidity) until ED20.

2.2.2 Sample collection

On ED20, eggs were opened and survivability was recorded for each treatment group. Live embryos were euthanized by cervical dislocation, individual body weight and yolk-free after yolk sac was removed) body weight taken to the nearest 0.01 g. Liver, spleen, and bursa were removed and weighed to the nearest 0.001 g. The small intestine was also removed, weighed and measured. The intestine was then split into its three consecutive segments: duodenum (attachment at gizzard to end of duodenal loop), jejunum (end of duodenal loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to ileo-cecal junction). Individual small intestine segments were weighed to the nearest 0.001 g and length measured to the nearest 0.1 cm. Density of intestinal segments was calculated as weight per unit length (g/cm). The whole spleen (n=15) and sections of the liver (n=15) were taken for histopathological analysis and fixed in 10% neutral buffered formalin. The liver section was taken from the bottom of the right lobe. After fixation in formalin for 72 hours, tissues were transferred and stored in 70% ethanol until histological processing. Whole spleen (n=15) and remaining liver tissue (n=15) were flash frozen in liquid nitrogen and stored at -80 ºC for subsequent TBARS assay (liver only) and gene expression analysis.

2.2.3 Tissue histology

Liver and spleen tissue from three treatment groups (CON, DMSO, DON 1.0, and DON 5.0; n=6) were trimmed to fit histopathological cassettes with a clean scalpel and submitted to Prairie Diagnostic Services Inc. (Saskatoon, Saskatchewan) for processing, sectioning and staining. Briefly, liver and spleen from each individual were paired, embedded in paraffin wax, and sectioned by microtome to a 5 μm thickness. Resulting sections were stained by hematoxylin and eosin (H & E) and mounted in triplicate on slides. Slides were examined blindly by an avian pathologist under an Axiostar Plus light microscope (Carl Zeiss Microscopy, LLC, One Zeiss Drive Thornwood, NY, USA) and evaluated for appearance of abnormalities, lesions and other structural changes. Micrographs were obtained using an Axiocam MRc (Carl Zeiss Microscopy)

and Axiovision. Rel. 4.8 software (Carl Zeiss Microscopy).

2.2.4 Measurement of lipid peroxidation levels

Malondialdehyde (MDA), a product of lipid peroxidation, was quantified in liver homogenates using the commercially available TBARS Parameter Assay Kit (R&D Systems, Abingdon, UK). In preparation, 100 mg liver samples from four treatment groups (DMSO, DON 0.2, DON 1.0 and DON 5.0; n=8) were homogenized, lysed using a Tris-Triton cell lysis solution, and acidified using the kit acid reagent to remove interfering proteins. The remainder of the assay was carried out per manufacturer's instructions. Briefly, acidified samples were centrifuged at 12 000 x g for 4 minutes and supernatant was removed. Samples and dilution standard were run in duplicate on a clear, 96-well plate: each well contained 150 µL of standard or sample and 75 µL of TBA reagent. Wells were pre-read for optical density at 532 nanometers (nm) (Epoch 2 Microplate Spectrophotometer, Bio-Tek Instruments Inc., Winooski, VT, USA). The plate was covered and incubated using a dry block heater (VWR International, Mississauga, ON, Canada) at 50 ºC for 2 hours, at which point the first plate reading was completed (532 nm). Additional readings were conducted between 2 and 3 hours of incubation and ceased when absorbance values began to decrease. Pre-readings were subtracted from final readings to quantify sample contribution to absorption. Concentration of MDA was calculated using the calibration curve generated through standard dilution readings (as per manufacturer instruction). MDA concentrations were corrected to account for dilution factor and tissue weight.

2.2.5 RNA isolation, reverse transcription and quantitative PCR

Liver and spleen tissue from four treatment groups (DMSO, DON 0.2, DON 1.0 and DON 5.0; n=8) were analyzed for expression of genes of interest relating to oxidative stress (AIFM1, BAX, CASP3, CASP9, and GPx4) and immunity (IL-6 and IL-8), respectively. Total RNA was isolated using TRIzol reagent (Ambion, Inc., Austin, TX, USA) as per manufacturer's instructions. RNA was extracted from 15 biological samples per tissue per treatment. The quantity and purity of RNA was assessed using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA) and the quality was verified on a 1% agarose gel. Following qualitative measurements, 10 of the total 15 samples/treatment were carried through to complementary DNA (cDNA) synthesis.

Total cDNA was prepared from 1 µg of total RNA using the Quantitect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) as directed by the manufacturer. Samples were diluted either 5- or 10-fold to obtain working concentrations prior to quantitative polymerase chain reaction (qPCR).

The expression of individual gene targets was analyzed by qPCR on a CFX96 Real-time C1000 Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). Gene-specific primers for transcripts specific to oxidative stress, apoptosis and immunity were sourced from the literature (Table 2.1). Reactions were run at a 20 µL volume consisting of 1µL SsoFast EvaGreen Supermix (Bio-Rad Laboratories), 0.8 µL of both forward and reverse primer (0.4 mM), and 2 µL input of sample cDNA at working concentrations. Samples were run in duplicate along with no-template control samples (RNase-free water instead of cDNA template) and a no-reverse-transcriptase control samples (cDNA template for which water is added instead of enzyme). A standard curve was run on each qPCR plate, made from a pooled cDNA samples and serially diluted. Standard curves for all primer pairs achieved amplification efficiency of 90-110% and an $R^2 > 0.98$. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. Duplicate data for each sample was averaged and normalized to the reference genes (GAPDH, 18S). Data was expressed as fold-change relative to DMSO control.

Table 2.1 Primers for real-time PCR gene expression analysis in domestic chicken (*Gallus gallus*)

Target	Name	Accession Number	Primer Sequence (5' to 3')	Tissue	Source
18S	18S ribosomal RNA	AF173612	**F:** CGAAAGCATTTGCCAAGAAT **R:** GGCATCTGTTTATGGTCGG	liver, spleen	Olias et al. 2014
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	AI981686	**F:** GGCACGCCATCACTATC **R:** CCTGCATCTGCCCATTT	liver, spleen	Antonissen et al. 2015
AIFM1	apoptosis inducing factor, mitochondria-associated 1	NM_001007490.1	**F:** GAAGTACAACAACGGCTGAC **R:** GAGACAGAGACAGACTTGAC	liver	Li et al. 2014
BAX	B-cell lymphoma 2 associated X protein	XM_422067.2	**F:** GTGATGGCATGGGACATAGCTC **R:** TGGCGTAGACCTTGCGGATAA	liver	Ren et al. 2015.
CASP3	caspase 3	NM_204725.1	**F:** TGGCCCTCTTGAACTGAAAG **R:** TCCACTGTCTGCTTCAATACC	liver	Li et al. 2014
CASP9	caspase 9	AY057940.1	**F:** GTGTACCAGCTGCGAGCAGACC **R:** GCTTTGAGGTTCCGCAGGGTCC	liver	Li et al. 2014
GPx4	glutathione peroxidase	AF498316	**F:** CAGTACAGGGGCTTCGTCTG **R:** CAGCCCCTTCTCAGCGTATC	liver	Haug et al. 2014
IL-6	interleukin 6	NM_204628.1	**F:** GAAATCCCTCCTCGCCAATCTGA **R:** GAAATCCCTCCTCGCCAATCTGA	spleen	Wang et al. 2012
IL-8	interleukin 8	AJ009800	**F:** ATGAACGGCAAGCTTGGAGCT **R:** TCACAGTGGTGCATCAGAATTGA	spleen	Kaiser et al. 1999

2.2.6 Statistical analyses

This study utilized a completely randomized design. Normality and homogeneity of variance were tested using the Shapiro-Wilk test and Levene's test, respectively. Data not meeting parametric assumptions were log transformed. Differences across treatment groups were determined using one-way analysis of variance (ANOVA) in SAS (Statistical Analysis Software, version 9.4, SAS Institute Inc., Cary, NC, USA). If main factor effects were significant, the Tukey-Kramer post-hoc test was used to differentiate means. For analysis of gene expression, Dunnett's post-hoc test was used for multiple comparison to DMSO control. A value of $P < 0.05$ was considered significant. All graphs were generated using Prism 6 (GraphPad Software, La Jolla, CA, USA).

2.3 Results

2.3.1 Embryo survivability and morphometrics

Percent survivability of embryos for each treatment group was as follows: CON = 96.7%, DMSO = 90.0%, DON 0.04 = 90.0%; DON 0.2 = 93.3%, DON 1.0 = 83.3% and DON 5.0 = 56.7%. DON treatment had no effect on total body weight at any dose; however, yolk-free body weight (YFBW) was decreased in the DON 5.0 group as compared to all other treatments (Table 2.2). Relative liver and spleen weights were increased in embryos from the DON 5.0 treatment group as compared to all other treatments (Table 2.2).

Livers from individuals in both the DON 1.0 and DON 5.0 treatment groups varied from light to dark green and spleens in the same individuals were visibly darkened and enlarged (Figure 2.1). This phenomenon appeared in 4% of surviving embryos in the DON 1.0 group and in 41% of surviving embryos in the DON 5.0 group. Weight, length and density of the intestinal segments (duodenum, jejunum, and ileum) were unchanged by any treatment. The weight of the bursa was also unaffected by treatment.

Table 2.2 Effect of *in ovo* injection of deoxynivalenol (DON, 0.04-5.0 μg/g egg weight) on survivability (%) and the mean (± S.E.) relative organ weights[1] (g) of Lohmann LSL x Bovan chicken embryos prior to hatch (ED20).

	Treatment				
	DMSO (vehicle)	**DON 0.04**	**DON 0.2**	**DON 1.0**	**DON 5.0**
Survivability	90.0	90.0	93.3	83.3	56.7
Body Weight	44.64 ±0.49	43.87 ±0.49	44.37 ±0.48	43.82 ±0.52	43.08 ±0.61
Yolk-Free Weight	35.23 ±0.42[a]	34.12 ±0.39[a]	34.35 ±0.39[a]	33.82 ±0.45[a]	30.74 ±0.50[b]
Organ Weights					
Liver	1.553 ±0.027[b]	1.554 ±0.027[b]	1.522 ±0.026[b]	1.562 ±0.028[b]	1.916 ±0.038[a]
Spleen	0.034 ±0.002[b]	0.030 ±0.002[b]	0.032 ±0.002[b]	0.030 ±0.002[b]	0.065 ±0.003[a]
Duodenum	0.260 ±0.008	0.276 ±0.008	0.271 ±0.008	0.249 ±0.009	0.244 ±0.010
Jejunum	0.441 ±0.013	0.440 ±0.013	0.461 ±0.013	0.447 ±0.014	0.401 ±0.018
Ileum	0.463 ±0.023	0.470 ±0.023	0.493 ±0.023	0.507 ±0.025	0.426 ±0.030
Bursa	0.132 ±0.007	0.121 ±0.007	0.128 ±0.006	0.134 ±0.007	0.114 ±0.008

[1]Relative organ weight = (absolute organ weight/body weight) x100
[a,b]Statistical difference following one-way ANOVA and differentiation of means by Tukey-Kramer post-hoc test ($p < 0.05$)

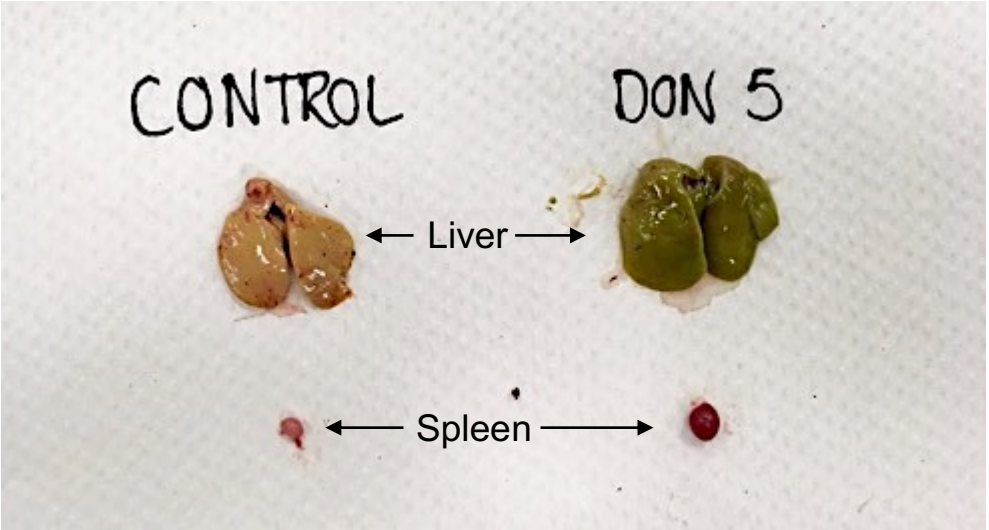

Figure 2.1 Comparison of liver and spleen gross pathology from an untreated chicken embryo (CON) and an embryo exposed to 5.0 µg DON/g egg weight (DON 5.0) prior to hatch (ED20) demonstrating hepatic bile stagnation and splenic inflammation.

2.3.2 Histopathology of liver and spleen

The liver tissue and spleens of randomly selected individuals (n=6) from CON, DMSO, DON 1.0, and DON 5.0 were H&E stained and histologically analyzed. Increased granulopoiesis, especially around portal areas, was evident in livers from DON 1.0 (Figure 2.2: C, G) and DON 5.0 (Figure 2.2: D, H) individuals as compared to both the untreated (Figure 2: A, E) and DMSO (Figure 2.2: B, F) control groups. Granulopoietic events appeared with greater frequency in the DON 5.0 group (Figure 2.2: D, H), as compared to DON 1.0, suggesting a dose-dependent response. Splenic histopathological changes were not associated with DON treatment (images not shown).

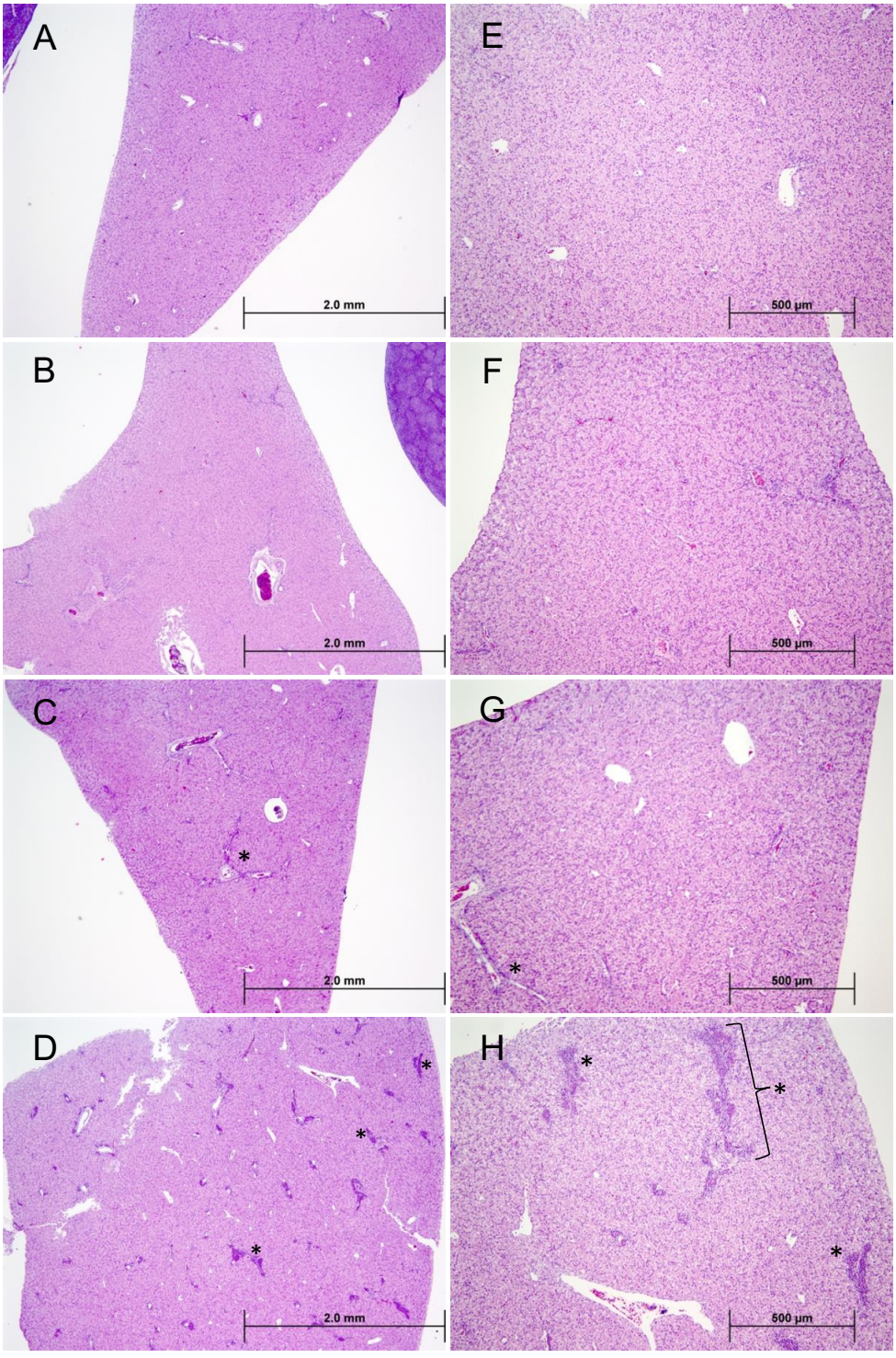

Figure 2.2 Photomicrographs of H&E stained liver sections from ED20 chicken embryos following *in ovo* exposure to increasing doses of DON. Side-by-side images were captured from single samples at 4X (A-D) and 10X (E-H) magnification. Images shown are from untreated control (A, E), DMSO (B, F), DON 1.0 (C, G) and DON 5.0 (D, H) treatment groups. Granulopoiesis (*) can be seen increasing in frequency as DON dose increases.

2.3.3 Hepatic lipid peroxidation

A single *in ovo* injection of DON resulted in a significant dose-dependent increased concentration of MDA in liver homogenates from chick embryos (Figure 2.3) across all treatment groups (n=8; Figure 2.3). Hepatic MDA production following DON 0.2, DON 1.0 and DON 5.0 exposure was approximately two, three, and four-fold greater than that of the DMSO control, respectively.

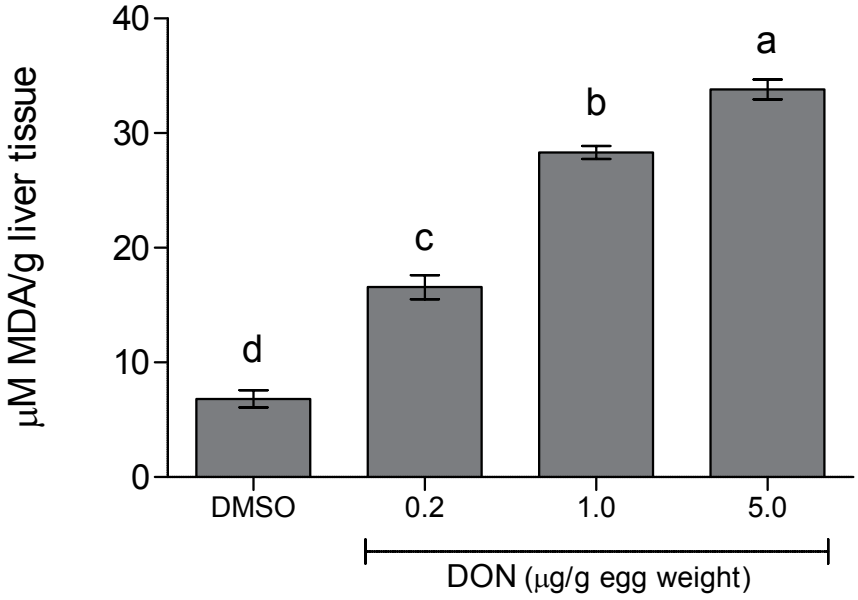

Figure 2.3 TBARS content in the chicken embryo liver following single *in ovo* exposure of DON at ED14. Values were generated using a standard dilution of known MDA concentrations and have been corrected to account for dilution factors and volume of tissue. Data are represented in bars (mean ± SEM). Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple pairwise comparisons. Significant differences ($p < 0.05$) are indicated by letter notation.

2.3.4 Gene expression in the liver and spleen

The relative mRNA expression of genes relating to apoptosis and oxidative stress (AIFM1, BAX, GPx4, CASP3 and CASP9) in the liver is shown in Figure 2.4. There was no effect of DON treatment on the expression of AIFM1, BAX, GPx4, and CASP3 (Figure 2.4: A-D). Embryos in the DON 1.0 group had significantly higher CASP9 expression in the liver as compared to DMSO group but there was no difference in CASP9 expression between DON 5.0 and DMSO (Figure 2.4E).

The relative mRNA expression of genes relating to immune processes (IL-6 and IL-8) in the spleen are shown in Figure 2.5. Expression of IL-6 was not affected by *in ovo* exposure to DON at any level (Figure 2.5A). Embryos in the DON 0.2 group had significantly higher IL-8 expression relative to DMSO (Figure 2.5B).

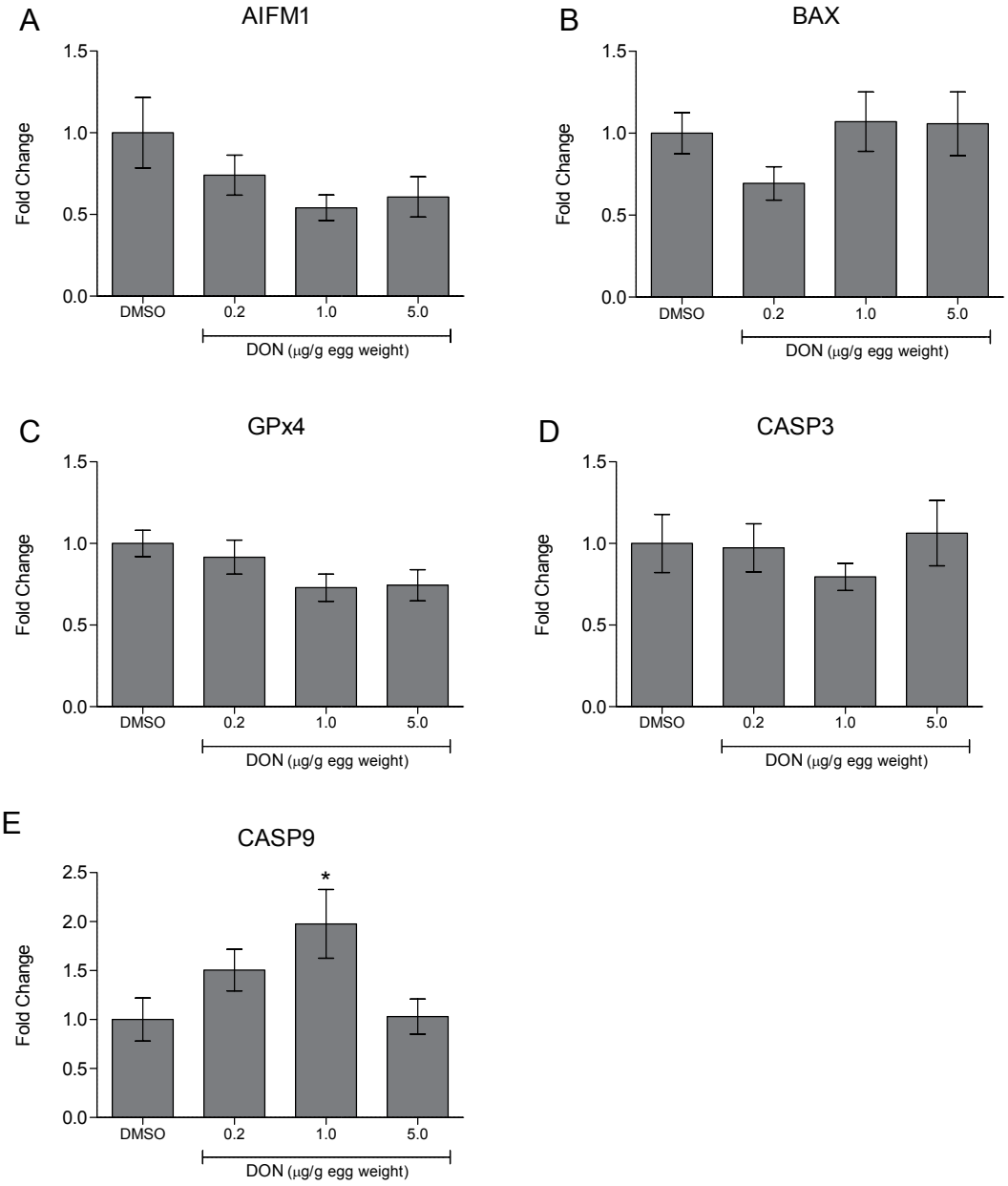

Figure 2.4 Liver mRNA expression of oxidative stress-related genes following *in ovo* exposure of chicken embryos to deoxynivalenol (DON). Data for each gene were normalized to the expression control genes (GAPDH and 18S) and expressed as fold change relative to vehicle control (DMSO). Bars represent gene expression (mean ± SEM). Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons to one control. Significant differences ($p < 0.05$) are indicated by *.

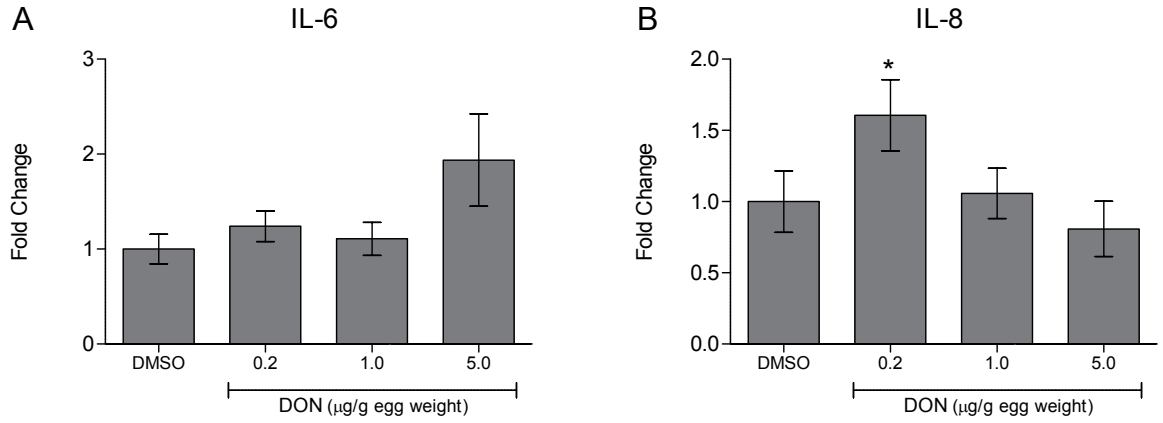

Figure 2.5 Spleen mRNA expression of IL-6 and IL-8 following *in ovo* exposure of chicken embryos to deoxynivalenol (DON). Data for each gene were normalized to the expression control genes (GAPDH and 18S) and expressed as fold change relative to vehicle control (DMSO). Bars represent gene expression (mean ± SEM). Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons to one control. Significant differences ($p < 0.05$) are indicated by *.

2.3 Discussion

Poultry consuming DON-contaminated diets commonly exhibit adverse physiological responses – most notably reduced feed intake, modified nutrient absorption, damage to the gut mucosa, hepatoxicity and immunosuppression or stimulation, depending on the level and duration of exposure. While systemic DON toxicity is most often studied using whole animal feeding studies, there may also be predictive value in using the chick embryo system relative to both *in vitro* and whole animal testing. In the present study, the chicken embryo, or *in ovo* model, was proposed as an intermediate between *in vitro* and *in vivo* toxicity testing of DON to better inform whole animal studies. As such, late-term chicken embryos were exposed via amniotic fluid injection to increasing concentrations of DON in solution. While not the first investigation of DON's effects *in ovo*, this work is the first to evaluate morphological, functional and molecular endpoints paralleling those commonly evaluated *in vivo*. Based on the results discussed below, the chicken embryo demonstrates a clear response to DON with effects on immunity and oxidative stress that are comparable to previous *in vitro* cell assays and *in vivo* studies with poultry.

In ovo exposure to the highest DON level at 5.0 µg DON/g egg weight resulted in toxic effects in the developing chick embryo that manifested in death. Embryo survivability was 56.7% for this group whereas the group exposed to 1.0 µg DON/g egg had 83.3% survivability and survivability for all other groups was 90% or greater. Increased mortality is not typically associated with dietary DON intake in the post-hatch chicken; for example, a 9-week exposure of Leghorn chickens to a high-level DON diet (18 mg/kg feed) did not increase mortality (Harvey et al., 1991). It is important to note that dietary exposures are based on DON per unit feed, whereas the majority of *in ovo* exposures are based on DON per unit egg weight, making it difficult to directly compare exposure concentrations. In the present study, DON solutions were administered directly into the amniotic fluid of the egg: the amniotic fluid is consumed by the embryo starting ED12, but consumption increases rapidly at ED14 – the time point at which treatment solutions of DON were administered. Therefore, to compare between studies, we must consider the amniotic fluid as the "feed" and convert the dose to DON per unit amniotic fluid. Assuming an egg weight of 65 g at set, an individual in the 5.0 µg DON/g egg weight treatment would have received a total of 325 µg of DON. A typical egg weight loss during incubation is approximately 0.3 g/day (Willems et al., 2014); thus, a 65 g egg would weigh 60.8 g at ED14. At this point, the amniotic fluid would compose about 30% (Willems et al., 2014) of the total egg mass (i.e. 18.24 g at ED 14). Based on these calculations, the concentration of DON in amniotic fluid for our 5.0 µg DON/g egg weight treatment group would be approximately 17.8 µg DON/g amniotic fluid or 17.8 ppm DON – considerably higher than the 5 ppm DON inclusion limit currently stipulated for poultry feed (CFIA, 2015; European Commission 2006; FDA 2012). Still, feeding DON at a similar level (18 mg/kg feed or 18 ppm; cited above) did not increase mortality in post-hatch birds, which suggests that exposure route and/or developmental differences in toxicokinetics contribute to disparities in the threshold levels for DON toxicity in the chicken embryo as compared to post-hatch birds. Within the gastrointestinal tract of adult chickens, DON is biotransformed to relatively non-toxic metabolites by the gut microbial population (Lun et al. 1988; He et al. 1992) as well as by enterocytes themselves (Schwartz-Zimmermann et al., 2015). Although toxicokinetics of DON have not been evaluated in the chicken embryo, it is hypothesized that detoxification of DON by these mechanisms *in ovo* is limited. The chicken egg and embryo are considered to be sterile, thus microbial biotransformation of DON is not a viable DON detoxification pathway in the chicken embryo. Additionally, the embryo enterocyte is structurally and functionally primitive and only begins to

resemble adult enterocytes in the days post-hatch (Geyra et al., 2001; Roto et al., 2016) and thus, has little capacity to contribute to DON detoxification. Taken together, the lack of biotransformation by the gut microbiota and within enterocytes likely increases chicken embryo exposure to injected DON and may, in part, explain the increased sensitivity of this model with respect to mortality.

In this study, yolk-free body weight decreased with DON administration while whole body weight (including yolk) was unchanged, indicating a larger yolk and smaller embryo with exposure to 5.0 μg DON/g egg weight. The yolk is a critical nutritional component for the developing embryo with approximately 90% of the total energy requirement of the chicken embryo fulfilled by beta-oxidation of yolk lipids (Noble and Cocchi, 1990); therefore, the development of the embryo pre- and post-hatch is highly dependent on the ability of the embryo to utilize the yolk (Yadgary et al., 2013). Our exposure period (ED14 – ED20) coincides with the last seven days of incubation – a stage characterized by high lipid demand wherein ~80% of the yolk-lipid is mobilized and absorbed to support rapid embryonic growth (Noble and Cocchi, 1990). Increased residual yolk and decreased embryo size observed in the present study suggests that DON exposure altered the ability of the chicken embryo to adequately absorb and/or metabolize the lipid-dense yolk. Inhibited yolk absorption may be the result of DON-induced hepatic bile stagnation. Green discoloration of the liver, indicative of substantial bile stagnation, was clearly visible in DON 1.0 and DON 5.0 individuals upon necropsy demonstrating a dose-dependent, cholestatic effect of DON on the chicken embryo. Typically, bile is produced in the liver and stored in the gall bladder then released into the small intestine in order to emulsify fats. Bile acids also appear in the yolk and yolk-sac membrane of the chicken embryo (Surai and Speak, 1998; Speake, 2006; Yadgary et al., 2013), serving as an emulsifier of yolk-lipids to aid in lipid uptake by the yolk-sac membrane. The origin of these bile acids is currently unknown; however, enterohepatic cycling with the yolk (Surai and Speak, 1998) and synthesis within the yolk-sac membrane (Yadgary et al., 2013) are suggested sources. Thus, cholestasis could impede the movement of bile into the yolk and ultimately reduce the absorption of yolk lipids. Measurement of hepatic lipid profiles could provide insight into mechanisms by which DON affects yolk utilization and embryo body weight - possibly via disruption of lipid metabolism. The effect of DON on lipid metabolism is not well-established, yet there is some evidence that feeding of DON-contaminated diets alters liver nutrient uptake in broilers (Dietrich et al., 2013) and lipid distribution in laying hens (Farnworth et al., 1983) and carp (Pietsch et al., 2014). In the

only other *in ovo* DON exposure to date, electron microscopy revealed an accumulation of fatty droplets in the liver of chicken embryos exposed to DON at ED12 (Moon et al., 2007). Moreover, hepatic total lipids in carp (Pietsch et al., 2014) and hepatic total cholesterol and triglycerides in laying hens (Farnworth et al., 1983) were significantly elevated following dietary exposure to DON. The chick liver accomplishes up to 95% of total fatty acid synthesis (O'Hea and Leveille, 1969) and is also a known target for DON in poultry. Given the reliance of the embryo on yolk lipids, altered lipid absorption and/or metabolism are hypothesized mechanisms for the morphometric changes and decreased survivability discussed above.

In addition to nutrient uptake and lipid distribution, the liver is responsible for detoxification of xenobiotics and is one of the first tissues to encounter ingested DON. In this study, chicken embryos exposed to the highest level of DON exhibited increased relative liver weight. Reported effects of DON on poultry organ weights, including the liver, are varied across studies and this is often attributed to differences in exposure concentration and duration (Swamy et al., 2004; Awad et al., 2011; Zain, 2011; Awad et al., 2013). A recent study found that consumption of low-level DON (< 5 mg/kg) contaminated feed increased liver weight in broilers (Yunus et al., 2012) but there was no mechanism proposed for this organ-level response. In the present study, histological analysis revealed a dose-dependent accumulation of granulocytes in livers of the DON 1.0 and DON 5.0 individuals. Granulopoietic events appeared with highest frequency and magnitude in the DON 5.0 group, suggesting that heavier livers are at least in part due to increased quantity of local granulocytes. Increased granulopoiesis following exposure to DON agrees with previous studies that report elevated circulating heterophils in broilers consuming DON (Levkut et al., 2011; Revajova et al., 2013). Heterophils are the most abundant avian granulocyte and are mobilized in response to chemotactic agents released during tissue damage at target organs (Maxwell and Robertson, 1998); therefore, damage to liver tissue may have promoted production of heterophils (*i.e.* granulopoiesis) observed in this study. Such tissue damage in the liver was evidenced in the dose-dependent increase in markers of lipid peroxidation (MDA) in response to DON exposure. Lipid peroxidation is a known mode of DON toxicity (Mishra et al., 2014) and was identified as the primary cause of liver damage in carp fed DON-contaminated diets (Pietsch et al., 2014). While increased liver granulopoiesis has not previously been observed in adult chickens fed DON-contaminated diets, increased lipid peroxidation is a widely reported response to DON-feeding in broiler chickens as well as cultured chicken cells exposed to DON *in vitro* (Borutova et al., 2008; Awad et al., 2014; Lautert

et al., 2014; Li et al., 2014; Ren et al., 2015). It is reasonable to infer that liver cell damage, due to lipid peroxidation, triggered granulopoiesis as both the granulopoietic and oxidative stress responses increased with DON concentration. There may also be a relationship between these cellular level effects and bile stagnation discussed earlier as liver damage is a known precursor for fat deposition and cholestasis.

While the measured increase in lipid peroxidation indicates an oxidative stress response in the liver, hepatic mRNA expression of apoptotic and oxidative stress genes was largely unaltered by DON exposure at any dose. The apoptotic and oxidative mechanisms of DON toxicity are well-established (Mishra et al., 2014) and expression of target genes are considered sensitive markers of oxidative stress (Osselaere et al., 2013); thus, upregulation of pro-apoptotic genes (AIFM1, caspase-3, caspase-9, BAX) and downregulation of antioxidant genes (GPx4) would be expected. Instead, only caspase-9, an initiator caspase, was upregulated in the livers of embryos administered 1.0 μg DON/g egg weight. Apart from this singular change, our results contradict previous *in vitro* reports which show increased expression, concentration and activity of pro-apoptotic proteins following DON exposure (Li et al., 2014; Ren et al., 2015; Benassi et al., 2012; respectively). Moreover, Li et al. (2014) also reported increased lipid peroxidation in conjunction with changes in gene expression. One possible reason for this discrepancy could be that changes in gene expression response do not always correlate well to physiological responses and often precede their onset following a chemical exposure. Time-dependent reduction in oxidative stress markers was previously reported in broiler lymphocytes following a single exposure to DON, suggesting cellular compensatory mechanisms (Lautert et al., 2014). Therefore, there may have been up- or downregulation of genes immediately following initial exposure that was not detected in tissues that were sampled six days later.

Increased spleen weight was observed in chicken embryos exposed to the highest level of DON. Upon necropsy, reddened and enlarged spleens indicative of splenic inflammation were observed in 41% of individuals in the same group. Splenic inflammation also occurred in the DON 1.0 group, but with reduced frequency (4%). In the chicken, the spleen is a principal organ of systemic immunity (John, 1994) and, like the liver, is a target for DON toxicity (Bondy and Pestka, 2000). Other studies have reported an increase in spleen weight in chickens fed DON-contaminated diets (Yunus et al., 2012; Chen et al., 2017) and DON-induced changes in spleen weight were also described to be dependent on exposure duration with swelling during acute feeding and shrinkage during chronic feeding (Swamy et al., 2004). In the present study,

exposure of the chick embryo to DON occurred over a relatively short period (six days; ED14 - ED20) and swelling and inflammation of the spleen corresponded to the increased spleen weight, thus, our findings are in line with previous observations in adult chickens. Interestingly, there were no histological alterations to spleen tissue associated with DON exposure. Chen et al. (2017) found that dietary consumption of DON increased the number of germinal centers in the chicken spleen. This specific response would not occur in the chicken embryo spleen since the secondary lymphoid functions of the spleen, such as antigen-dependent lymphocyte differentiation, only begin post-hatch (John, 1994). Instead, the embryonic spleen undertakes granulopoiesis (John, 1994) and an immunostimulatory effect of DON would likely be directed toward this process. The spleen is closely linked to the liver through circulation in that blood is supplied to the spleen through the coeliac artery and drains into the liver from the larger hepatic portal vein (John, 1994). Considering the increased number of granulocytes seen in the liver, it is possible that DON stimulated granulopoiesis in the spleen and that splenic granulocytes were subsequently transported to the liver in response to DON-induced oxidative damage.

Within the spleen, interleukin-6 (IL-6) expression was unaffected by DON treatment, which was surprising given the observed splenic inflammation and the ability of DON to modulate cytokine production *in vivo* (reviewed in Awad et al., 2013). It is important to note that, in this case, IL-6 was very lowly expressed and results were highly variable between individuals, perhaps making it difficult to discern DON-specific effects. Expression of interleukin-8 (IL-8) changes were also unexpected as upregulation only occurred at the second lowest DON dose administered (DON 0.2). This, while unexpected again, agrees with the putative dose-dependent immune effects of DON being immunostimulatory effects at low doses and immunosuppressive effect at higher doses (Pestka, 2008).

In summary, our results demonstrate that the chicken embryo responds to DON exposure through changes related to immunity and the induction of hepatic oxidative stress. Specifically, we report that DON induces tissue- and cellular level responses in the embryonic liver and the spleen. It appears that DON's effects in the chicken embryo are comparable to those reported in adult birds fed DON-contaminated diets, especially with regards to lipid metabolism, hepatic oxidative stress, and inflammatory immune response Based on these results, we propose the chicken *in ovo* model could be used as an alternative to study mechanisms of DON toxicity in poultry and, in the future, may be used in this capacity to predict whole animal responses to emerging mycotoxins or relevant mycotoxin combinations. Importantly, the chicken embryo

appears to be more sensitive to DON and comparative levels of DON *in vivo* and *in ovo* should be taken into consideration when using the *in ovo* model as a predictive tool.

CHAPTER 3

INDIVIDUAL AND COMBINED EFFECTS OF *FUSARIUM* MYCOTOXINS, DEOXYNIVALENOL AND ZEARALENONE, FOLLOWING *IN OVO* EXPOSURE IN THE LATE-TERM CHICKEN EMBRYO

PREFACE

In the previous chapter, we determined that physiological and pathological responses to the mycotoxin *in ovo* were conservatively predictive of *in vivo* DON toxicity. The objective of the research presented in Chapter 3 was to characterize the combined toxicity of DON and ZEA, administered *in ovo*, to the chicken embryo and compare responses in the chick embryo to those observed in previous combinatorial studies. As such, single injections of high (2.5 μg/g egg weight) and low (0.5 μg/g egg weight) doses of purified DON and/or ZEA were administered to late-term chicken embryos. Our results demonstrate that interactive effects of DON and ZEA differed across endpoints, varying from antagonistic at low doses to non-interactive or possibly potentiated at high doses. Additionally, independent effects of DON were consistent with those observed in Chapter 2.

Author contributions:

Émilie Viczko (University of Saskatchewan) helped design the experiment, conducted the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Natacha Hogan (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and obtained funding for the research.

3.1 Introduction

The increasing occurrence of *Fusarium* fungi and associated mycotoxins in cereal grains is a significant issue for global agriculture. While *Fusarium* infection impacts the yield and quality of grains, consumption of mycotoxin-contaminated grains by livestock reduces overall productivity and health, resulting in significant economic losses to both grain and animal industries (Hussein et al., 2001). At present, up to 400 different mycotoxins have been identified and among this group exists a wide array of chemical structures and unique toxic effects (Berthiller et al., 2013). A confounding factor in the risk of mycotoxin toxicity is the fact that multiple mycotoxins, produced by one or more fungal species, can accumulate in individual feed commodities that compose a single diet, ultimately resulting in co-occurrence of different mycotoxins within feeds (Smith et al., 2016). A recent, world-wide survey of feeds and raw feed ingredients identified found that 71% of 18,757 samples tested contained more than one mycotoxin (Biomin, 2017). Based on recent meta-analyses of literature reporting mycotoxin occurrence in foods and feeds, the combination of deoxynivalenol (DON) and zearalenone (ZEA) is considered to be the most prevalent mycotoxin mixture in North America (Smith et al., 2016) and world-wide (Streit et al., 2012).

Dietary exposure to DON and ZEA individually is a significant health concern for animals and humans. As an estrogenic mycotoxin, ZEA maintains structural similarity to estrogen and, therefore, competes with 17β-estradiol for binding to the estrogen receptor, consequently interfering in fertility and reproduction (Metzler et al., 2010). Beyond its estrogenic impacts, numerous *in vitro* studies have reported ZEA's capacity to induce oxidative stress (Abbid-Essefi et al., 2004; Abbid-Essefi et al., 2009; El Golli-Bennour et al., 2008; Bouaziz et al., 2008; Lee et al., 2013). DON is a member of the trichothecene family and ingestion of DON-contaminated feed can cause anorexia, vomiting, and impaired immunity in various production animals (reviewed in Eriksen and Petterson, 2004). Within the cell, DON binds to ribosomes and inhibits protein synthesis through a process known as the ribotoxic stress response. This response can lead to apoptosis through direct activation of mitogen-activated protein kinases (reviewed in Pestka et al. 2007), or through oxidative stress pathways (Mishra et al., 2014). The capacity of DON to inhibit protein synthesis and induce apoptosis can significantly affect tissues requiring rapid cell turnover, such as the gut, liver, and immune tissues and cells (Bondy and Pestka, 2000; Pinton and Oswald, 2014).

While many studies report the individual toxicity of mycotoxins, in practice, the outcome of combined exposure may be qualitatively or quantitatively different from what would be predicted based on individual toxicity (Speijers and Speijers, 2004). Simultaneous exposure to multiple mycotoxins can result in antagonistic, additive or synergistic toxicological effects (reviewed in Alassane-Kpembi et al., 2017). Despite the frequency of DON and ZEA co-contamination, little is known about their toxic interactions. Thus far, *in vitro* evidence suggests that DON and ZEA together act additively and/or synergistically but have limited estrogenic effects. Swine jejunal epithelial cells exposed to a combination of DON and ZEA displayed decreased cell viability despite individual doses being non-cytotoxic (Wan et al., 2013). The effects of aflatoxin B_1 (AFB) and DON together, as well as AFB and ZEA, on rat liver cells were synergistic in terms of oxidative stress indicators (Sun et al., 2015). Although this study did not explore the combination of DON and ZEA, similar hepatotoxic effects when combined with AFB suggest overlapping toxicity and potential for interaction. Regarding *in vivo* effects, dietary exposure of broilers to the combination of DON and ZEA at high (8.2 mg/kg + 8.3 mg/kg) and low (3.4 mg/kg + 3.4 mg/kg) concentrations significantly increased oxidative stress in the liver and decreased circulating lymphocyte and phagocyte populations (Borutova et al., 2008; Levkut et al., 2011). Unfortunately, in both studies, the singular and combined effects of DON and ZEA were not contrasted because treatment groups exposed to DON or ZEA alone were not included. In a mouse feeding study, DON and ZEA individually induced oxidative stress in the mouse kidney and, combined, their nephrotoxic effect was concluded to be sub-additive (Liang et al., 2015); however, the interaction effect was not specifically evaluated in the statistical analyses.

Studying mycotoxin interactions is notably complex and often require numerous treatment groups, as well as large sample sizes, to perform adequate dose-response analysis. The majority of mycotoxin mixture work is conducted *in vitro*, rather than *in vivo*, as cell models are less restrictive in the number of test groups (Alassane-Kpembi et al., 2017). There are also current ethical concerns regarding animal-based toxicity testing, which are driving the research and regulatory communities to find alternatives that reduce animal use (Rovida et al., 2015). Unfortunately, *in vitro* methodology fails to capture systemic interplay and using *in vitro* data to predict whole animal responses can be challenging. Considering the limitations of both *in vitro* and *in vivo* research, alternative models for combinatorial mycotoxin research should be explored. Studies using chicken embryos could be transitional between *in vitro* studies on cell cultures and *in vivo* experiments on whole organisms. Delivery of toxicants *in ovo* still results in

whole animal exposure, while the nutritional self-sufficiency of the chicken embryo combined with direct administration ensures a controlled dosing scenario, reducing the variability associated with mycotoxin feeding studies. Past studies have successfully used early embryo assays to define individual and combined toxicity of maternally-deposited mycotoxins (aflatoxin, ochratoxin, citrinin) (Edrington et al. 1995; Veselá et al. 1983; Oznurlu et al. 2012; Saleemi et al. 2015; Monson et al. 2016). Previous research demonstrated that *in ovo* administration of DON to the late stage chicken embryo induced responses that were comparable to those reported for post-hatch birds, specifically in terms of oxidative stress and immunotoxicity (Chapter 2). This approach may therefore also be useful for assessing adverse responses to mycotoxin mixtures, allowing researchers to rapidly characterize mycotoxin interactions and prioritize combinations for further assessment in whole animals.

The aim of the present study was to characterize the combined toxicity of DON and ZEA administered *in ovo* to the chicken embryo and compare responses in the chick embryo to those observed in previous combinatorial studies. To this end, embryo and organ morphology, tissue structure of the liver and spleen, and hepatic lipid peroxidation were evaluated to determine the whole organism, tissue-level and functional responses to DON and ZEA alone and in combination.

3.2 Materials and Methods

3.2.1 Eggs and treatment procedure

Three-hundred ten fertilized Lohmann LSL x Bovan (female x male) eggs were obtained from the University of Saskatchewan layer flock and housed at the Poultry Research Centre at the University of Saskatchewan. Prior to the start of incubation (set), individual egg weight was recorded. Set began within 24 hours of lay for all eggs and was considered embryonic day 1 (ED1). From ED1 to ED18, the incubator was maintained at 37.5 ºC and 60% humidity. On ED14, eggs were candled and those that were infertile or contained early dead embryos were removed.

Purified DON (CAS # 51481-10-8; purity $\geq$ 98%) and purified ZEA (CAS #17924-92-4; $\geq$ 98%) were purchased from Cedarlane (Burlington, ON, Canada) and supplied by Toronto Research Chemicals Inc. (North York, ON, Canada). Dimethyl sulfoxide (DMSO; CAS # 67-68-5) was purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Four individual

solutions containing purified DON or purified ZEA were prepared at concentrations of 0.5 and 2.5 µg/µL in 20% DMSO. Two combined solutions of purified DON and purified ZEA were prepared at concentrations of 0.5 µg DON + 0.5 µg ZEA/µL and 2.5 µg DON + 2.5 µg ZEA/µL in 20% DMSO. Each aforementioned solution was administered to the corresponding treatment group at a volume of 1 µL/g of egg weight as measured on ED1. Treatments (n=30) included an untreated control group, a vehicle-injected control group (20% DMSO), 0.5 and 2.5 µg DON/g egg weight, 0.5 and 2.5 µg ZEA/g egg weight, 0.5 µg DON + 0.5 µg ZEA/g egg weight, 2.5 µg DON + 2.5 µg ZEA/g egg weight and will hereby be referred to as CON, DMSO, 0.5 DON, 2.5 DON, 0.5 ZEA, 2.5 ZEA, 0.5 DON + 0.5 ZEA, and 2.5 DON + 2.5 ZEA, respectively. Prior to solution injection, egg surfaces were disinfected with a 70% ethanol solution. A diamond-tipped Dremel tool was used to drill a hole in the air cell end of each egg. Solutions were drawn using a 200-µL pipette fitted with a gel-loading tip (VWR; Cat # 37001-152), the tip was inserted into the hole and through the membrane at an ~45º angle and the solution was released into the amniotic fluid of the egg. Pipette tips were changed between each injection. Holes were then sealed with silicon (GE Silicon I, all-purpose, clear) and eggs were returned to the incubator, positioned air sac up, until ED18. On ED18 the eggs were transferred to a second incubator at 37.5 ºC and 70% humidity until ED20.

3.2.2 Sample collection

Eggs were opened on ED20 and the survivability of each treatment group was recorded. Surviving embryos were then euthanized by cervical dislocation and the individual body weights with and without the yolk (yolk-free body weight) were taken to the nearest 0.01 g. The weights of the liver, spleen, and bursa were taken to the nearest 0.001 g. The small intestine was removed and split into segments as described in Chapter 2. The weights of the individual segments were taken to the nearest 0.001 g and the same segments were length measured to the nearest 0.1 cm. Density of each segment was determined by calculate weight per unit length (g/cm). From each treatment group, whole spleens (n=15) and sections of the liver (bottom of right lobe; n=15) were taken for histopathological analysis. Tissues were fixed for ~ 72 hours in 10% neutral buffered formalin after which time they were transferred into 70% ethanol and stored until processing. Liver tissue remaining after histological sampling was flash frozen in liquid nitrogen and stored at -80 ºC until further analysis.

3.2.3 Liver and spleen histology

Processing, sectioning, mounting and staining of tissues was conducted at the Histology Core Facility at the University of Saskatchewan. Briefly, liver and spleen tissue from four treatment groups (DMSO, 2.5 DON, 2.5 ZEA, 2.5 DON + 2.5 ZEA; n=6) were trimmed to fit histopathological cassettes. The liver and spleen from each individual were paired within the cassettes, subsequently processed, embedded in paraffin and sectioned at a 5 μm thickness. Non-consecutive sections were mounted in triplicate on slides and stained by hematoxylin and eosin. Slides were examined blindly under an Axiostar Plus light microscope (Carl Zeiss Microscopy, LLC, One Zeiss Drive Thornwood, NY, USA). Micrographs were obtained using an Axiocam MRc (Carl Zeiss Microscopy) and AxioVision. Rel. 4.8 software (Carl Zeiss Microscopy). Histological examination of the liver and spleen focused on the detection of granulopoiesis and bile duct proliferation as these tissue-level changes were previously observed in DON exposed embryos (Chapter 2). Briefly, the quantity and size of granulopoietic events and number of bile duct proliferations were determined in three independent fields per section at 10x magnification. Identity of granulocytes and bile duct proliferations were confirmed at 100x magnification with an oil-immersion lens. Based on these findings, severity of each parameter in the liver and spleen was scored: 0 – none, 1 – minimal, 2 – mild, 3 – moderate, or 4 – severe. After a period of time, slides were blindly re-evaluated to validate initial findings.

3.2.4 Measurement of hepatic lipid peroxidation

TBARS, specifically MDA, were measured in liver homogenates using the commercially available TBARS Parameter Assay Kit (R&D Systems, Abingdon, UK). The assay was conducted twice on two separate plates – one containing samples from the DMSO (n=5) and low dose (0.5 DON, 0.5 ZEA, 0.5 DON + 0.5 ZEA; n=8) treatment groups and the other containing samples from the DMSO (n=5) and high dose (2.5 DON, 2.5 ZEA, 2.5 DON + 2.5 ZEA; n=8) treatment groups. Briefly, liver samples of ~100 mg from six treatment groups (DMSO, 0.5 DON, 0.5 ZEA, 0.5 DON + 0.5 ZEA, 2.5 DON, 2.5 ZEA, 2.5 DON + 2.5 ZEA) were homogenized, lysed with a Tris-Triton cell lysis solution, and acidified using the kit acid reagent. Acidified samples were centrifuged at 12 000 x g for 4 minutes and the supernatant was removed. Samples and standard were run in duplicate on clear, 96-well plates. The plates were covered and incubated using a dry block heater (VWR International, Mississauga, ON, Canada)

at 50 ºC for two hours, at which point the first reading was completed at 532 nm using the Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). Additional readings were conducted in half-hour intervals until absorbance readings decreased. The concentration of MDA in individual samples was calculated using the formula generated from the standard curve and then corrected for dilution factor and tissue weight.

3.2.5 Statistical analyses

Data were analyzed in a 2 x 2 factorial design with responses to DON and ZEA (individually and combined) analyzed separately at the low dose (0.5 ug/g egg weight) and the high dose (2.5 ug/g egg weight). Normality and homogeneity of variance were tested using the Shapiro-Wilk test and Levene's test, respectively. Two-way ANOVA in SAS (Statistical Analysis Software, version 9.4, SAS Institute Inc., Cary, NC, USA) was used to determine if interaction effects between DON and ZEA were significant. If interaction effects or main factor effects were significant, the Tukey-Kramer post-hoc test was used to differentiate means. A value of $P < 0.05$ was considered significant. Interaction graphs were generated using Microsoft Excel.

3.3 Results

3.3.1 Embryo survivability and morphometrics

Percent survivability of embryos for each treatment group was as follows: CON = 97%, DMSO = 97%, 0.5 DON = 93%, 0.5 ZEA = 90%, 0.5 DON + 0.5 ZEA = 97%, 2.5 DON = 93%, 2.5 ZEA = 97% and 2.5 DON + 2.5 ZEA = 80%.

Effects of *in ovo* injection of low doses of deoxynivalenol (0.5 DON) and zearalenone (0.5 ZEA) alone and together, on mean relative organ weights are shown in Table 3.1. Body weight, yolk-free body weight, and organ weights of the spleen and bursa were not significantly different across treatment groups. Relative liver weight was significantly higher in individuals in the 0.5 DON treatment groups as compared to the vehicle control (DMSO) and 0.5 DON + 0.5 ZEA groups. Relative liver weight in individuals treated with 0.5 ZEA alone did not differ from any other treatment. An interactive effect was confirmed ($F = 10.18$, $p = 0.0019$) indicating an antagonistic effect of ZEA on response to DON in terms of relative liver weight. Weight, length

and density of the intestinal segments (duodenum, jejunum, and ileum) were not significantly different between treatments.

Effect of *in ovo* injection of high doses of deoxynivalenol (2.5 DON) and zearalenone, (2.5 ZEA) alone and together, on mean relative organ weights are shown in Table 3.2. There were no significant differences in body weight or in the weight, length, and density of intestinal segments across treatment groups. However, there was a significant main effect of DON on yolk-free body weight ($F = 10.84$, $p = 0.0014$). Yolk-free body weight in groups receiving 2.5 DON (2.5 DON, 2.5 DON + 2.5 ZEA) was significantly decreased as compared to groups receiving no DON (DMSO, 2.5 ZEA). There was no interactive effect of DON and ZEA on relative liver weight but there was a main effect of DON treatment ($F = 10.43$, $p = 0.0017$) and ZEA treatment ($F = 14.46$, $p = 0.0002$). Relative liver weight was significantly higher in individuals administered 2.5 DON compared to those not exposed to DON while individuals administered 2.5 ZEA also had higher liver weight compared to those not receiving ZEA. There was also a main effect of DON ($F = 8.77$, $p = 0.0038$) and ZEA ($F = 4.54$, $p = 0.0355$) on relative spleen weight with increased relative spleen weight in groups exposed to DON or ZEA as compared to those not receiving DON or ZEA. Hepatic bile stagnation paired with splenic inflammation was observed in 14% and 25% of surviving embryos from the 2.5 DON and 2.5 DON + 2.5 ZEA groups, respectively (data not shown). There was also a significant main effect of ZEA on bursa weight ($F = 4.44$, $p = 0.0376$). Relative bursa weight in groups receiving 2.5 ZEA (2.5 ZEA, 2.5 DON + 2.5 ZEA) were significantly decreased as compared to groups not receiving ZEA (DMSO, 2.5 DON).

Table 3.1. Effect of *in ovo* injection of low doses of deoxynivalenol (DON, 0.5 µg/g egg weight) and zearalenone (ZEA, 0.5 µg/g egg weight) alone and together on survivability (%) and the mean relative organ weights[1] (g) of Lohmann LSL x Bovan chicken embryos prior to hatch (ED20).

DON, µg/g egg wt	**0**		**0.5**			**P-value**		
ZEA, µg/g egg wt	**0**	**0.5**	**0**	**0.5**	**SEM**	**DON**	**ZEA**	**DON x ZEA**
Survivability	96.7	90.0	93.3	96.7				
Body Weight	43.80	43.09	43.05	43.83	0.31	0.9871	0.9465	0.1730
Yolk-Free Weight	33.71	33.34	33.43	33.21	0.23	0.6719	0.5291	0.8819
Organ Weights								
Liver	1.523[b]	1.561[ab]	1.659[a]	1.526[b]	0.015	0.0601	0.0792	0.0019
Spleen	0.030	0.028	0.031	0.029	0.002	0.4790	0.6839	0.5831
Duodenum	0.260	0.264	0.277	0.253	0.004	0.7132	0.3121	0.1061
Jejunum	0.512	0.525	0.534	0.476	0.009	0.2981	0.7673	0.1033
Ileum	0.488	0.515	0.526	0.465	0.011	0.7470	0.3669	0.1030
Bursa	0.124	0.131	0.131	0.143	0.005	0.1312	0.1645	0.9929

[1]Relative organ weight = (absolute organ weight/body weight) x100

[a,b]Statistical difference following two-way ANOVA and differentiation of means by Tukey-Kramer post-hoc test ($p < 0.05$)

Table 3.2. Effect of *in ovo* injection of high doses of deoxynivalenol (DON, 2.5 µg/g egg weight) and zearalenone (ZEA, 2.5 µg/g egg weight) alone and together on survivability (%) and the mean relative organ weights[1] (g) of Lohmann LSL x Bovan chicken embryos prior to hatch (ED20).

DON, µg/g egg wt	**0**		**2.5**			**P-value**		
ZEA, µg/g egg wt	**0**	**2.5**	**0**	**2.5**	**SEM**	**DON**	**ZEA**	**DON x ZEA**
Survivability	96.7	96.7	93.3	80.0				
Body Weight	43.80	43.35	42.70	43.06	0.32	0.2179	0.9285	0.4737
Yolk-Free Weight	33.71	33.47	32.59	31.74	0.28	0.0014	0.2134	0.4823
Organ Weights								
Liver	1.523	1.593	1.579	1.701	0.015	0.0017	0.0020	0.3120
Spleen	0.032	0.034	0.036	0.044	0.001	0.0038	0.0355	0.1667
Duodenum	0.260	0.271	0.261	0.254	0.004	0.3626	0.7948	0.2405
Jejunum	0.512	0.510	0.475	0.518	0.009	0.4199	0.2411	0.2043
Ileum	0.488	0.498	0.489	0.485	0.010	0.7591	0.8974	0.7222
Bursa	0.124	0.116	0.148	0.121	0.005	0.0723	0.0376	0.2555

[1]Relative organ weight = (absolute organ weight/body weight) x100

3.3.2 Histopathology of liver and spleen

Liver and spleens in individuals (n=6) from the DMSO treatment group and the high mycotoxin groups (2.5 DON, 2.5 ZEA and 2.5 DON + 2.5 ZEA) were randomly selected for histopathological analysis. Histopathological examination of the liver did not reveal abnormalities (Figure 3.1). There were no major changes in liver tissue structure observed between the 2.5 ZEA or DMSO; however, increased granulopoiesis was observed in the 2.5 DON group with a higher density of granulocytes appearing near portal areas (Table 3.3; 3.4). In the 2.5 DON + 2.5 ZEA group, frequency of granulopoietic events were increased as compared to DMSO, 2.5 ZEA or 2.5 DON alone (Table 3.3; 3.4). Regarding the spleen, there were no histopathological changes associated with treatment (images not shown).

Table 3.3 Scheme utilized for visual assessment and scoring of hepatic granulopoiesis in ED20 chicken embryos following *in ovo* injection high doses of deoxynivalenol (2.5 µg/g egg weight) and zearalenone (2.5 µg/g egg weight) alone or in combination.

Score	Histopathological Parameters
0 – None	No evident concentrations of granulocytes
1 – Minimal	2 – 5 small areas of concentrated granulocytes
2 – Mild	5 – 10 small areas or 1 – 3 large areas of concentrated granulocytes
3 – Moderate	10 – 15 small areas or 3 – 5 large areas of concentrated granulocytes
4 – Severe	>15 small areas or > large areas of concentrated granulocytes

Table 3.4 Hepatic granulopoiesis scores[1] of ED20 chicken embryos following *in ovo* injection of high doses of deoxynivalenol (DON, 2.5 µg/g egg weight) and zearalenone (ZEA, 2.5 µg/g egg weight) alone or in combination.

DON, µg/g egg wt	**0**		**2.5**	
ZEA, µg/g egg wt	**0**	**2.5**	**0**	**2.5**
1	1	0	3	3
2	0	1	2	3
3	1	0	3	3
4	0	1	1	3
5	1	1	2	3
6	1	1	1	3
Median	**1**	**1**	**2**	**3**

[1]Scoring was based on the scheme outlined in Table 3.3

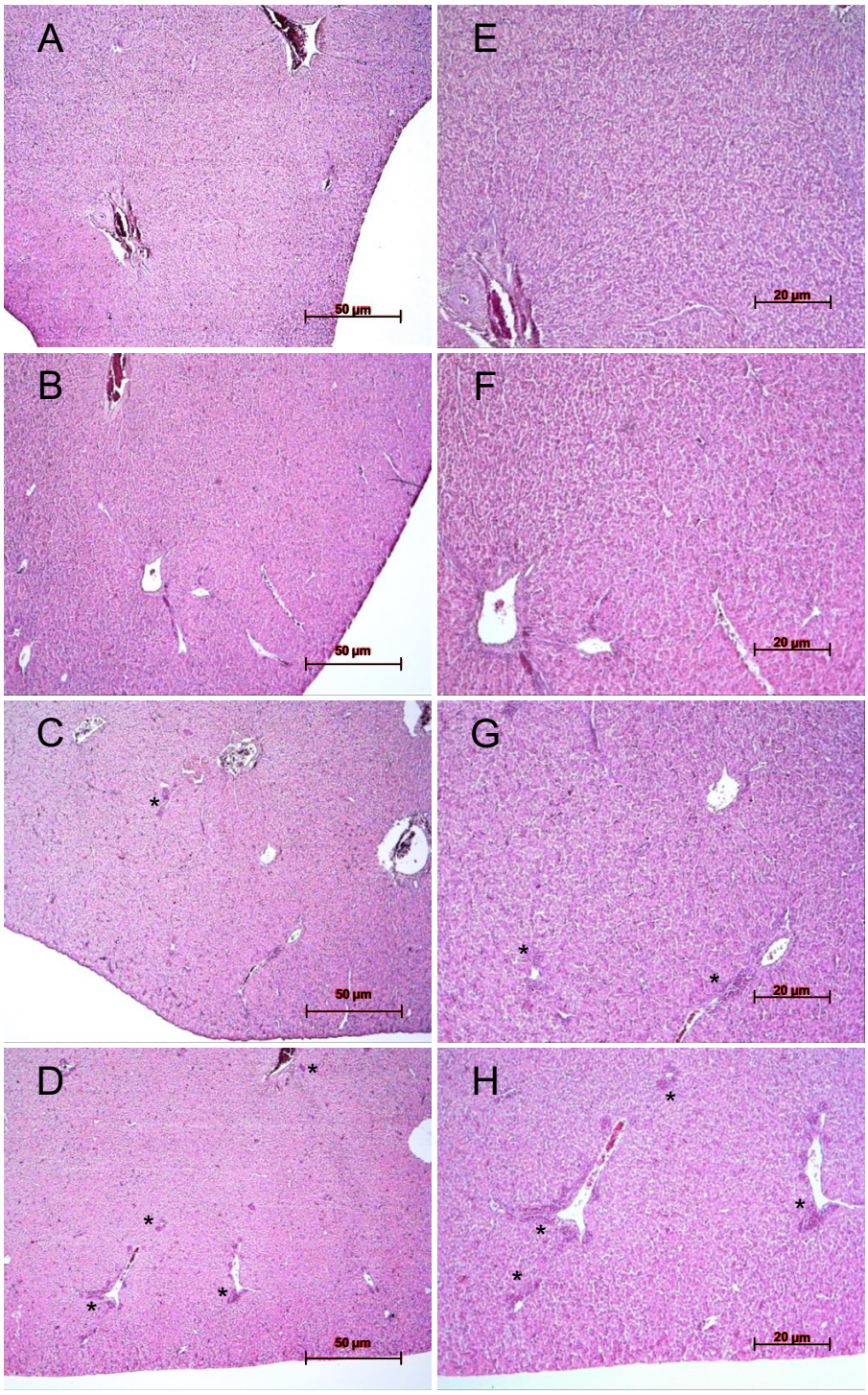

Figure 3.1 Photomicrographs of H&E stained liver sections from ED20 chicken embryos following *in ovo* exposure to 2.5 µg DON/g egg weight and/or ZEA. Side-by-side images were captured from single samples at 5X (A-D) and 10X (E-H) magnification. Images shown are from DMSO (A, E), ZEA (B, F), DON (C, G) and 2.5 DON + 2.5 ZEA (D, H) treatment groups. Granulopoiesis (*) appears with increased frequency in the DON (C, G) and 2.5 DON + 2.5 ZEA (D, H) groups.

3.3.3 Hepatic lipid peroxidation

The effect of administration of ZEA and DON alone, and their interactions, on hepatic MDA is shown in Figure 3.2. There was an interactive, antagonistic effect of 0.5 DON + 0.5 ZEA on liver MDA content ($F = 8.26$, $p = 0.0074$; Figure 3.2A). Hepatic MDA content was significantly higher in the 0.5 ZEA group as compared to the combined dose, 0.5 DON + 0.5 ZEA, but neither group differed significantly from DMSO or 0.5 DON. There was no main effect of either DON or ZEA at this level. While there was no interactive effect of 2.5 DON and 2.5 ZEA, there was a main effect of DON ($F = 8.70$, $p = 0.0065$) on hepatic MDA content (Figure 3.2B). Liver MDA content was significantly higher in groups receiving 2.5 DON (2.5 DON, 2.5 DON + 2.5 ZEA) as compared to those receiving no DON (DMSO, 2.5 ZEA).

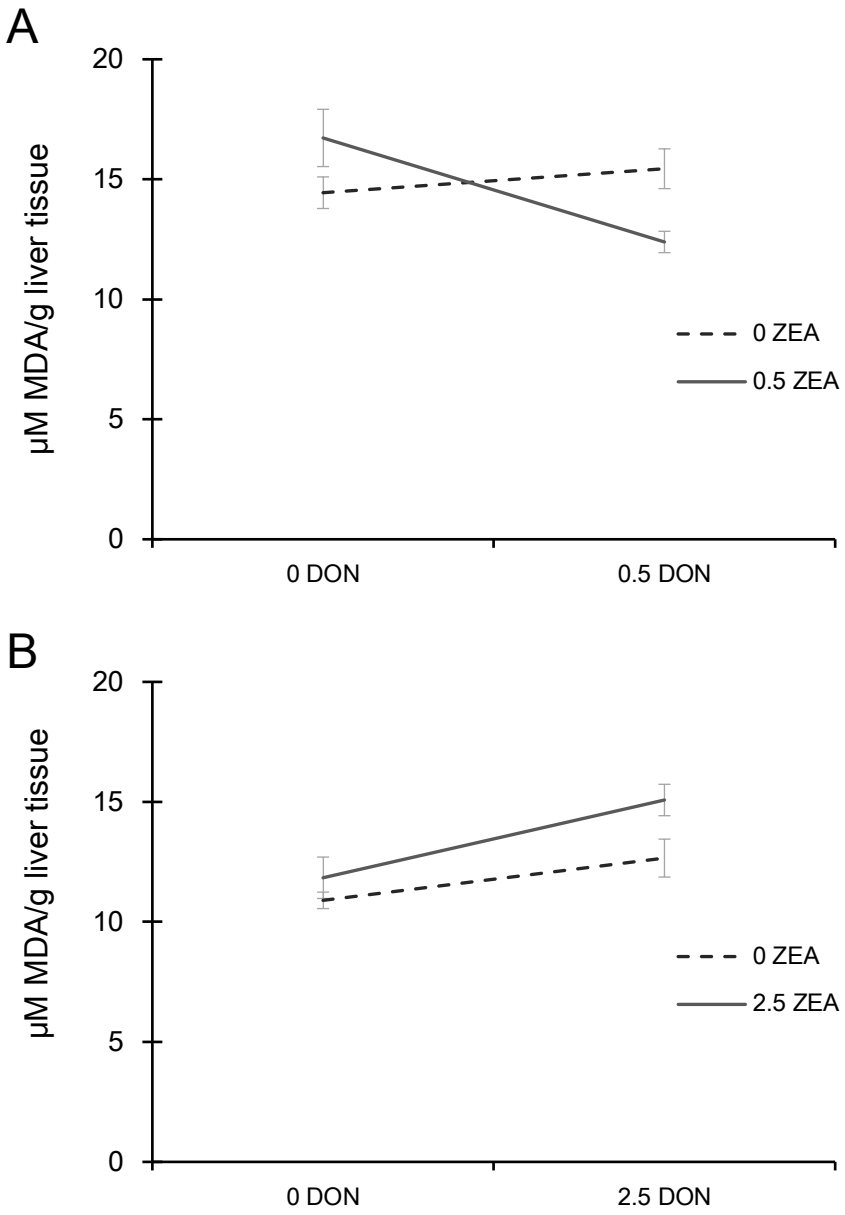

Figure 3.2 Hepatic MDA content (an indicator of lipid peroxidation) in the chicken embryo liver following a single *in ovo* exposure to 0.5 µg DON/g egg weight and/or ZEA (A) or 2.5 µg DON/g egg weight and/or ZEA (B). Values were generated using a standard dilution of known MDA concentrations and were corrected to account for dilution factors and volume of tissue. Ends of lines represent mean ± S.E.M. Data were analyzed using two-way ANOVA to determine interactive effects followed by Tukey-Kramer post-hoc test for separation of means.

3.4 Discussion

Despite the frequency of mycotoxin co-contamination in feed, very few mycotoxin interactions have been characterized *in vivo* due to limitations of whole animal studies. The chicken embryo, or *in ovo* model, shows value as a conservative predictor of whole animal responses to DON (Chapter 2) and, therefore, could also be used to predict responses to mycotoxin mixtures. The present study aimed to characterize the combined toxicity of DON and ZEA *in ovo* and to compare responses in the chick embryo to those reported in other combinatorial studies. To this end, chicken embryos were exposed to two concentrations of DON and ZEA, individually and combined, via amniotic fluid injection. Endpoints that displayed clear responses to DON alone in previous work (Chapter 2) were evaluated in the present study. Our results demonstrate that interactive responses to DON and ZEA depend on the endpoint measured and range from antagonistic at low doses to non-interactive or possibly potentiative at high doses.

At high concentrations, the combined administration of DON and ZEA was overtly toxic to the developing chicken embryo, resulting in embryonic death. Embryo survivability for the 2.5 DON + 2.5 ZEA group was 80% while survivability for all other treatment groups was 90% or greater, including groups receiving 2.5 DON and 2.5 ZEA alone. The fact that the combination of DON and ZEA elicited a greater response than either individual mycotoxin at the same dose suggests that DON and ZEA, at high doses, may be potentiative regarding chicken embryo mortality. There are no prior reports in poultry where dietary exposure to DON and ZEA, alone or in combination, affected survival. In fact, exposure to very high concentrations of DON (70 mg DON/kg body weight; Huff et al., 1981) or ZEA (800 mg ZEA/kg feed; Chi et al., 1980) did not increase mortality of poultry. Additionally, survival was not affected in mice fed high levels of DON (25 mg/kg) and ZEA (10 mg/kg) combined in feed (Pestka et al., 1987). In Chapter 2, *in ovo* exposure to DON alone decreased survivability of chicken embryos. It was hypothesized that the increased mortality was related to a lack of DON detoxification as well as DON-induced disruption in yolk absorption as the chicken embryo is highly dependent on yolk lipid utilization (Noble and Cocchi, 1990; Yadgary et al., 2013). In the present study, yolk-free body weight was lowered in groups receiving DON (2.5 DON and 2.5 DON + 2.5 ZEA) as compared to groups not receiving DON (DMSO and 2.5 ZEA), yet whole-body weight was not altered. This indicates that decreased embryo weight was offset by residual or unabsorbed yolk, suggesting that DON also inhibited or altered yolk utilization in this study. Moreover, hepatic bile stagnation,

demonstrated by green discolouration of the liver, was apparent in 2.5 DON-exposed groups but occurred more frequently in the group receiving 2.5 DON + 2.5 ZEA (25%) than the group receiving 2.5 DON alone (14%). While this observation is consistent with our previous findings (Chapter 2), it is interesting that bile stagnation was detected more often at a combined, high dose than either high dose of DON or ZEA individually. This again suggests that there may be potentiation between DON and ZEA at a high dose. It is hypothesized that bile, originating in the embryonic liver, moves into the yolk contents to emulsify yolk lipids during the final days of incubation (Surai and Speak, 1998; Speake, 2006). Thus, DON-induced (and ZEA-potentiated) cholestasis would impede bile movement into the yolk, decreasing absorption and potentially decreasing embryo weight or impacting embryo survival.

In the present study, there was no interaction of DON and ZEA at the high dose on organ weights; however, both DON and ZEA alone increased liver weight of the developing chicken embryo. Increased liver weight has been reported in poultry with dietary exposures to DON (Yunus et al., 2012) and ZEA (Jiang et al., 2011). In our previous study, we found that *in ovo* exposure to DON increased liver weight and that livers displayed increased granulopoiesis (production of granulocytes) as observed through histological assessment (Chapter 2). In the present study, substantial hepatic granulopoiesis was again observed in individuals exposed to 2.5 DON + 2.5 ZEA, as well as 2.5 DON alone, and so increased liver weight could be attributed to increased granulocyte production within the tissue. Granulopoietic events occurred more frequently in the combined exposure than with exposure to 2.5 DON or 2.5 ZEA alone, suggesting a potential potentiative effect of DON and ZEA at a high dose. Both DON and ZEA have been reported to stimulate immune cell production with *in vivo* exposure. Lymphoid infiltration of the liver, specifically, was observed in mice (Abbès et al., 2006a) and swine (Jiang et al., 2010) fed ZEA-contaminated diets, while broilers consuming DON-contaminated feed had increased number of circulating heterophils (Levkut et al., 2011; Revajova et al., 2013). When fed together, DON and ZEA increased the number of lymphocytes, plasma cells, and macrophages in the gastrointestinal lamina propria of gilts (Lewczuk et al., 2016). Heterophils, an abundant avian granulocyte, are highly responsive to chemotaxis and are among the first immune cells to arrive at damaged tissues (Maxwell and Robertson, 1998). We found that DON alone increased the MDA concentration in liver tissue, indicating potential liver damage by means of lipid peroxidation. Indeed, lipid peroxidation has previously been identified as the primary cause of liver damage in carp fed DON-contaminated diets (Pietsch et al., 2014).

Heterophils and other granulocytes may have been stimulated by DON-induced tissue damage resulting in the observed hepatic granulopoiesis. Furthermore, liver damage is a known precursor for cholestasis, thus, DON-induced tissue damage may have also contributed to the hepatic bile stagnation previously discussed.

Unlike DON, ZEA's effect on liver weight is not supported by the histopathological or biochemical findings. The ability of ZEA to increase liver weight independently may instead be attributed to its estrogenic effects. Estrogens enhanced the ability of various growth factors to stimulate DNA synthesis in cultured female rat hepatocytes (Ni and Yager, 1994). As previously mentioned, ZEA is structurally similar to estrogen and is able to bind estrogen receptors to elicit estrogenic effects. Through an estrogenic mechanism of action, ZEA may have enhanced liver growth by stimulation of growth factors.

A high dose of ZEA alone did not alter markers of lipid peroxidation despite that fact that lipid peroxidation is a well-documented response to ZEA both *in vitro* (Abid-Essefi, 2004; Abid-Essefi, 2009; Lee et al., 2013; Lautert et al., 2014) and *in vivo* (Jiang et al., 2011; Zhou et al., 2015). The lack of effect of ZEA alone on liver granulopoiesis and lipid peroxidation may be related to the dose of ZEA used. Poultry are considered to be somewhat resistant to DON, yet they are considerably more resistant to the effects of ZEA. For example, regulatory guidelines are provided for DON inclusion in poultry feed, while governing bodies do not currently impose dietary limits for ZEA in poultry (CFIA, 2015; European Commission 2006; FDA 2012). Here, DON and ZEA were administered at equal concentrations and while the dose of DON administered incited a clear, toxic response, the equivalent dose of ZEA may not have been sufficient to elicit toxic interactive or independent effects with respect to granulopoiesis or lipid peroxidation.

In the present study, exposure to the high concentration of DON, independent of ZEA, resulted in increased spleen weight. Red and enlarged spleens, indicative of splenic inflammation, were clearly visible upon necropsy in the 2.5 DON (14%) and 2.5 DON + 2.5 ZEA (25%) exposure groups. These organ-level changes are consistent with our previous findings (Chapter 2) and can likely be attributed to an inflammatory response incited by DON at low doses (Swamy et al., 2004; Chapter 2). Spleen weight was also elevated, and bursal weight decreased in groups receiving 2.5 ZEA (2.5 ZEA and 2.5 DON + 2.5 ZEA) as compared to those not receiving 2.5 ZEA (DMSO and 2.5 DON). To date, these effects on spleen and bursa have not been reported in poultry with *in vivo* exposure to ZEA and could be related to the

estrogenicity of ZEA. The immune system is sensitive to estrogenic compounds, in part due to the presence of estrogen receptors in immune cells (Igarashi et al., 2001). Estrogenic compounds can be immunostimulatory or immunosuppressive depending on the dose and timing of exposure. In chickens, the spleen is a principal organ of systemic immunity (John, 1994) and exposure to ZEA has been shown to alter mRNA expression of cytokines in chicken splenic lymphocytes (Wang et al., 2012) as well as increase acetylcholinesterase activity (involved in inflammatory responses) in broiler lymphocytes (Lautert et al., 2014). While immunostimulation by ZEA may have resulted in an increase in spleen weight, ZEA is also less estrogenic than endogenous estrogens (Metzler et al., 2010) perhaps explaining the absence of observable histopathological changes. The bursa, another avian immune organ, is also estrogen-sensitive. In particular, the embryonic bursa displays increased numbers of estrogen-responsive cells during the last week of incubation (Shin et al., 2012) which coincides with our treatment period (ED14 – ED20). It is possible that the decrease in bursa weight is related to the estrogenicity of ZEA; however, mechanisms underlying this morphological change remain unknown.

At a combined low dose, DON and ZEA elicited an interactive, antagonistic effect on relative liver weight. Exposure to DON alone increased relative liver weight when compared to the vehicle control, yet, when DON and ZEA were administered together, this effect was diminished. Feeding diets with a combination of DON and ZEA induced hepatic oxidative stress in broilers (Borutova et al., 2008) and liver tissue changes in swine (Tiemann et al., 2008; Weaver et al., 2014; Gerez et al., 2015), and these findings along with the results of the present study indicate that the liver is a target for the combined toxicity of DON and ZEA. We found that a low, combined dose of DON and ZEA also had an antagonistic effect on lipid peroxidation as indicated by decreased hepatic MDA concentration. A similar antagonistic effect of DON and ZEA on hepatic MDA was reported in mice following a two-week exposure via oral gavage (Sun et al., 2014). Given the relationship between hepatic MDA and relative liver weight, it is likely that changes in liver weight are related to oxidative effects of DON and ZEA. DON is well-known to elicit oxidative stress through its action at the mitochondria (reviewed in Mishra et al., 2014). While exact mechanisms of ZEA-induced oxidative stress are unknown, mitochondrial dysfunction and subsequent induction of apoptosis have been implicated in ZEA cytotoxicity to chicken splenic lymphocytes (Wang et al., 2016). Therefore, it is possible that, at low concentrations, DON and ZEA act competitively at the mitochondria and suppress one another's toxic effects, producing an antagonistic response with respect to oxidative stress.

It is interesting to note that the combined effects of DON and ZEA differ based on the dose of the mycotoxins administered. Here, responses range from antagonistic at the low, combined dose to non-interactive or possibly potentiative at the high, combined dose. In their review of combinatorial *in vivo* exposures, Grenier and Oswald (2011) determined that effects of mycotoxin mixtures on performance were generally additive or synergistic, but effects on other endpoints, including biochemical parameters, were often different for the same combination. Interaction dynamics similar to those in the present study have been observed following combined DON and ZEA exposures *in vitro*. Boiera et al. (2000) determined that the effect of DON and ZEA, together, on growth of brewing yeast differed by dose: at high doses DON and ZEA behaved synergistically while antagonism was observed at a lower dose. In contrast, the combined effect of DON and ZEA on cell viability of swine jejunal epithelial cells was synergistic at individually non-cytotoxic doses, but antagonistic at cytotoxic doses (Wan et al., 2013). Neither of these studies could offer mechanistic explanations for these differences, likely because the understanding of ZEA's toxic mechanisms beyond estrogenicity are limited.

In conclusion, our results demonstrate that interactions between DON and ZEA differ across endpoints measured and tend to vary from antagonistic at low doses to non-interactive or possibly potentiative at high doses. At low doses, DON and ZEA have antagonistic effects on liver weight as well as hepatic lipid peroxidation. At high doses, effects of DON and ZEA are mostly independent and effects of DON, specifically, are in line with our previous research. At a combined, high dose of DON and ZEA there is evidence of possible potentiation with respect to embryo survivability, hepatic bile stagnation and splenic inflammation, and hepatic granulopoiesis. It is important to note that suggested potentiation is purely observational. While the factorial analysis applied here is among the most robust statistical analyses for *in vivo* interaction studies (Alassane-Kpembi et al., 2017), it does not allow us to differentiate responses beyond interactive (synergistic or antagonistic) or non-interactive. Based on these results, the chicken *in ovo* model may be useful in studying the combined toxicity of mycotoxins, however, responses to the combination of DON and ZEA in this model, as well as other *in vitro* and *in vivo* models, are still variable. Further research regarding the mechanisms of ZEA-induced toxicity may improve the understanding of the responses observed here and in other studies.

CHAPTER 4

GENERAL DISCUSSION

4.1 Project Overview and Summary of Findings

Fusarium fungi and their toxic secondary metabolites, known as mycotoxins, are prevalent contaminants of cereal crops worldwide. Mycotoxins are readily found in finished feed and can elicit toxic effects such as reduced growth performance, altered gut health and compromised immunity, when ingested. Regulatory agencies have attempted to limit mycotoxin exposure in livestock by setting legislated or recommended limits for individual mycotoxins in feed (CFIA, 2015; European Commission 2006; FDA 2012). These limits, however, do not consider that a single feed source may contain a mixture of mycotoxins whose combined toxicity is likely to be additive or synergistic. At present, data on systemic effects of co-occurring mycotoxins are limited making it difficult to adjust current in-feed mycotoxin limits. The chicken embryo, or *in ovo* model, shows potential as screening tool for characterizing the systemic toxicity of mycotoxins alone and in combination, yet very few studies have employed *in ovo* methodology in the study of mycotoxins or mycotoxin interactions. In this thesis research, two *in ovo* exposures were conducted to determine the value of the *in ovo* model as a predictive tool for assessing the individual and combined toxicity of *Fusarium* mycotoxins.

In the first experiment, the effects of *in ovo* administration of DON were evaluated. Results indicated that the chicken embryo responds to *in ovo* DON exposure with effects on immunity and oxidative stress that are supported by previous *in vivo* and *in vitro* findings. At the highest dose, DON decreased embryo survivability and increased the relative weights of the embryonic liver and spleen. Hepatic bile stagnation and concurrent splenic inflammation were also frequently detected following administration of the highest and second highest DON dose but were observed less often in the latter. A dose-dependent increase in granulopoiesis and lipid peroxidation were observed in the liver; however, mRNA expression of genes related to immune and oxidative stress were mostly unchanged. The *in ovo* approach developed and validated in the first experiment was then carried forward to a second experiment with the aim of characterizing the combined toxicity of DON and another mycotoxin, zearalenone (ZEA) administered *in ovo* to the chicken embryo. Based on the results, it was concluded that the responses to the combination of DON and ZEA were variable. Interactions between DON and ZEA differed across endpoints from antagonistic at low doses to non-interactive or possibly potentiative at high doses. At a combined low dose, DON and ZEA had an antagonistic effect on embryonic liver weight as well as hepatic lipid peroxidation. At a combined high dose, effects of DON and ZEA were mostly independent and effects of DON, specifically, were in line with the previous exposure to DON

alone. At the combined, high dose of DON and ZEA there appeared to be evidence of potentiation with respect to embryo survivability, hepatic bile stagnation and splenic inflammation, and hepatic granulopoiesis.

4.2 The *in ovo* model as an alternative to *in vivo* animal testing

The ethical concern surrounding whole animal toxicity testing is driving researchers to find alternatives that reduce their animal use where possible. At present, factorial design analysis remains the most robust approach for studying combinatorial mycotoxin toxicity *in vivo*. An important drawback of this approach, however, is that it requires many test groups and large sample sizes to account for the inherent variability surrounding *in vivo* exposures. Sources of variability including feed (feed composition, unidentified substances in feed) and the quantity of toxin ingest are difficult to control and can also contribute to a lack of reproducibility between seemingly similar studies. Reducing animal use in science is guided by three principles: replacement, reduction, and refinement. Importantly, our results demonstrate that *in ovo* responses to mycotoxins, alone and in combination, are conservatively predictive of *in vivo* responses. While *in ovo* experiments are unlikely to replace *in vivo* animal trials, they can help to reduce the number of animals used for mycotoxin toxicity testing. *In ovo* exposure allows for a precise dosing scenario wherein mycotoxins are directly administered into the amniotic fluid of the egg and are consumed entirely by the embryo during the last phase of incubation. Because the nutrients are already supplied to the embryo through egg components (yolk, amniotic fluid/albumen), this reduces variability associated with as well as quantity of ingest. Moreover, reduced variability and isolation within the egg means individual embryos/eggs can function as test units rather than groups (i.e. cage, pen, or tank) which further reduces animal use. Therefore, the *in ovo* model could be used in an integrated approach where it acts as an intermediate between *in vitro* and *in vivo* study to identify and prioritize emerging mycotoxins and high-risk mycotoxin combinations for further *in vivo* assessment. This would allow whole animals to be used only when necessary, ultimately reducing the total number of animals utilized for mycotoxin toxicity research.

Beyond reducing animal usage, assessing mycotoxin toxicity *in ovo* can reduce costs associated with *in vivo* testing as *in ovo* models are relatively resource-conservative. To conduct an *in vivo* exposure, researchers must first purchase and transport whole animals which, depending on the species used, can be expensive. Housing space for the animals must also be

procured prior to their arrival. Again, depending on the species used, space requirements can be substantial and housing for any species must comply with production standards and/or stipulations set out by internal animal care committees. Stipulations may include environmental specifications for stocking density, airflow, water supply and quality, temperature control, lighting periods, and cages and bedding. A space that has both environmental controls and adequate capacity for whole animal may not be accessible for all researchers. In contrast, the space requirements for *in ovo* trials are minimal. During our exposures, chicken embryo/egg units remained within a temperature and humidity-controlled incubator that required little to no maintenance. Embryo/egg units were only removed once during the incubation period for the purposes of candling and treatment. While our trials were carried out in the university hatchery building, similar trials could be conducted within most lab spaces as benchtop incubators are becoming widely accessible. Moreover, low-cost, fertilized eggs can be obtained from commercial farms or university poultry science units (Henshel et al., 2002). The nutritional self-sufficiency of chicken embryos is perhaps the most cost-effective aspect of the *in ovo* model. Feed can be a significant expense for *in vivo* mycotoxin exposures. Researchers must purchase a supply of both clean and contaminated feed or feed ingredients that will last through the exposure duration. Once feed has been obtained, researchers must be able to test feed or feed ingredients for mycotoxin content and use this information to formulate feeds of known mycotoxin concentration. Feed formulation becomes even more challenging with combinatorial mycotoxin studies as feed contaminated with individual mycotoxins as well as the combination must be produced. *In ovo* exposures can greatly improve the efficiency of combinatorial mycotoxin exposure. In our studies, injection solutions containing individual mycotoxins and their combination were created by dissolving crystallized mycotoxins of known purity in 10% DMSO. This easily allowed us to control the dose and ratio of mycotoxins in exposure solutions.

4.3 Advantages and limitations of the *in ovo* model for mycotoxin screening

As previously discussed, *in vivo* responses to mycotoxins are difficult to reproduce often leading to under- or over-estimation of mycotoxin toxicity. Lack of reproducibility among *in vivo* studies can also increase animal usage as multiple studies attempt to assess mycotoxin toxicity across similar endpoints (i.e. growth performance, organ morphology etc.). This thesis research demonstrates that *in ovo* responses to the mycotoxin, DON, are both repeatable and reproducible. Across both experiments, *in ovo* exposure to DON resulted in significant and

consistent morphological alteration including decreased embryo survivability, decreased yolk utilization (yolk-free body weight), and increased weight and physical changes in the liver and spleen. Effects of mycotoxins on morphological traits tend to vary greatly amongst *in vivo* studies and, in general, are considered insensitive markers of *in vivo* toxicity (Ghareeb et al., 2012). Beyond morphology, exposure to DON in both experiments resulted in increased hepatic granulopoiesis and lipid peroxidation. Repeatability and reproducibility are measures of experimental accuracy. Our data, being both repeatable and reproducible, confirms the precision of *in ovo* mycotoxin exposure and indicates that our findings are an accurate representation of *in ovo* DON toxicity. Factors that may have contributed to the precision of our experiments should be addressed. Firstly, fertilized eggs for both exposures were supplied by the same flock, thus, genetic influence was consistent. Secondly, by removing the embryos from the egg prior to hatch, we removed variability associated with rapid growth immediately post-hatch.

It is important to note that use of the *in ovo* model does not constrain experimental design. Despite the small size of the chicken embryo (which permits minimal space requirements), ample tissue can be collected allowing for various types of analyses. For example, in both experiments, liver tissue from a single embryo was sufficient for histopathological, biochemical (TBARS), and gene expression analysis. Use of the chicken embryo also allowed a full systemic dose-response to be studied. In Chapter 2, we were able to determine a dose-response relationship for DON toxicity in the chicken embryo and understanding the effect of dose on toxicity of mycotoxins is crucial for statistical analysis of individual and combined mycotoxin toxicity.

Metabolic differences between the chicken embryo and the adult chicken are one of the main limitations for extrapolating results from an *in ovo* exposure to the whole animal. In both studies, *Fusarium* mycotoxins were administered into the amniotic fluid of fertilized eggs in order to mimic a dietary route of exposure. However, contrasting our findings with previous *in vivo* studies suggests that there may be fundamental differences in mycotoxin uptake and metabolism that modify responses to mycotoxins. For instance, the gut is a metabolically active tissue and, as such, is known to be susceptible to DON toxicity (Pinton and Oswald, 2014). Poultry consuming DON-contaminated diets (5 mg DON/kg feed) typically have lower intestinal segment weight and altered gut mucosal structures (e.g. reduced villi height; Awad et al., 2006); however, we did not see changes in weight, length and density of the small intestine. We did not histologically evaluate mucosal structures in the intestine based on a previous pilot study, where

chicken embryos exposed late in incubation (ED18) did not exhibit changes in weight or density of the small intestine or in the intestinal mucosal structures of the duodenum, jejunum or ileum. Although it is possible that histological, biochemical or gene expression changes occurred in the intestine with *in ovo* exposure to DON, based on the present research, it remains unknown whether the characteristic gastrointestinal effects of DON exposure would be manifested in the chicken embryo.

The limited role of microbiota within the gastrointestinal tract of the chicken embryo could be regarded as either an advantage or limitation of *in ovo* mycotoxin toxicity testing. The sterility of the chicken embryo gastrointestinal tract allows the effect of mycotoxins to be discerned independent of microbial metabolism; however, absence of microbiota does not reflect the situation in a post-hatch bird. It is understood that the microbial population of the gut can affect the mycotoxin toxicity as some common gut microbes are capable of metabolizing mycotoxins to detoxified forms. Moreover, microbial detoxification of DON is considered to be an important factor in poultry's resistance to DON (Lun et al. 1988; He et al. 1992). It was hypothesized that, in the present thesis research, a lack of microbial detoxification actually contributed to the increased sensitivity of the chicken embryo to DON and the combination of DON and ZEA. Therefore, without microbial influence, *in ovo* responses to mycotoxins may only conservatively predict the impact of mycotoxin exposure in the post-hatch bird and may, in fact, be exaggerated in comparison.

Another potentially limiting factor for *in ovo* mycotoxin research is the use of purified mycotoxins as the contaminant source. First, purified mycotoxins can be very expensive and certain mycotoxins that are classified as human poisons (e.g. T-2 toxin) may be difficult to obtain due to legislated health and safety regulations. Second, and perhaps more importantly, mycotoxins are a diverse group of compounds with many different structures that facilitate differing solubilities. When conducting an *in ovo* combinatorial exposure with purified mycotoxins, researchers must consider the individual solubilities of the mycotoxins and select a non-toxic vehicle in which they can deliver the combination of mycotoxins. Researchers must also be aware of the solution volume they are injecting into the egg as >100 µl of an aqueous solution injected into the egg environment can cause toxin-independent mortality (based on a 50-60 g egg; DeWitt et al., 2005). Thus, some mycotoxin mixtures may not be feasibly tested *in ovo*, as differences in solubility (i.e. polar or non-polar) may necessitate separate vehicles for individual mycotoxins and, thus, increased injection volume.

4.4 Future Directions

Overall, the late-term chicken embryo appears to be a conservative indicator of *in vivo* responses to *Fusarium* mycotoxins alone and in combination. An important factor in understanding toxicity of any compound is understanding the systemic toxicokinetics of that compound. Indeed, previous research studies investigating the toxicokinetics of DON and ZEA have greatly improved our understanding of poultry resistance to these toxins. At present, no information is available regarding toxicokinetics of DON and ZEA within the chicken embryo. This is not surprising, given that neither DON (Valenta and Dänicke, 2005) nor ZEA (Dänicke et al., 2002; Sypecka et al., 2004) are maternally deposited in the egg environment and thus, chicken embryos are not exposed to these mycotoxins in a production setting. However, this information is critical for comparing toxic effects between the chicken embryo and the adult chicken. It is currently unknown how DON and ZEA are absorbed, distributed, metabolized and eliminated by the embryo or whether effects of these mycotoxins reported in the current thesis research are due to the parent compounds or other bioactivated metabolites that may not appear in the adult chicken. Although our results demonstrate similar responses to previous *in vivo* and *in vitro* work, we cannot confirm that DON and ZEA are eliciting toxicity via the same compound. Future research should be directed towards understanding the toxicokinetics of mycotoxins within the chicken embryo as this information would help determine the accuracy with which the chicken *in ovo* model predicts the *in vivo* situation.

As previously discussed, purified mycotoxins are primarily used during *in ovo* mycotoxin exposure. While this allows researchers to evaluate the toxicity of individual mycotoxins and controlled ratios of mycotoxin combinations, it does not reflect the naturally-occurring mycotoxin matrix. Indeed, naturally-contaminated feed is generally considered more toxic than artificially contaminated sources (Eriksen and Petterson, 2004), which is likely due to interaction of masked, previously undescribed or co-occurring mycotoxins interacting with known mycotoxins (Schatzmayr and Streit, 2013; Dersjant-Li et al., 2003; Trenholm et al., 1994). Future research aimed toward isolating a mycotoxin matrix from grain could improve the applicability of the *in ovo* exposures with respect to mycotoxin exposure. Hypothetically, isolated mycotoxins extracted from a grain sample could be crystallized, resuspended in an appropriate vehicle, retested to determine mycotoxin concentrations and administered to the chicken embryo in the same way purified mycotoxins were applied in this thesis research. This would result in an *in ovo* exposure that more closely resembles a practical, dietary exposure. In

this way, the *in ovo* model might also be used as a rapid screening tool for feed where a mycotoxin matrix from a grain sample could be evaluated for toxicity using an effects-directed approach.

In an effort to address the growing concern of mycotoxin contamination in feed, appreciable research has been directed toward to development of mycotoxin mitigating products. In general, these products are aimed at reducing the toxic effects of mycotoxins by binding the mycotoxins, blocking mechanisms of action or through detoxification. Mitigation agents can be divided into three distinct groups – physical, chemical and biological methods of mitigation. Physical methods including quick drying, UV treatment, floating, near-infrared transmittance are typically focused on treatment of grains that reduces the amount or availability of mycotoxins prior to feeding (Zhu et al., 2016). However, chemical and biological methods are aimed at detoxification or compensation during the feeding process (Zhu et al., 2016). Given that the chicken embryo demonstrates adverse responses to mycotoxins, there may be potential for chemical and biological methods of mitigation to be trialed *in ovo*. Mitigation products applied in tandem or subsequent to mycotoxin injection could allow researchers to preview mitigation product efficacy and to use this information to better inform future *in vivo* trials. Moreover, this type of evaluation could enable privately-owned biotechnology companies to improve approval processes for new mycotoxin mitigating agents. This function could ultimately add to the industrial relevancy of the *in ovo* model as feeding programs could be adapted to include effective, mycotoxin mitigating additives based, in part, on data generated from *in ovo* research.

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