# IMPACT OF SEX AND AGE ON DEOXYNIVALENOL-INDUCED ANOREXIA USING A MURINE MODEL

By

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## ABSTRACT

IMPACT OF SEX AND AGE ON DEOXYNIVALENOL-INDUCED ANOREXIA USING A MURINE MODEL

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The trichothecene mycotoxin deoxynivalenol (DON, vomitoxin) is produced as a secondary metabolite by the fungus *Fusarium graminearum* and commonly contaminates grains including corn, wheat, and barley. DON is highly resistant to heat processing and can enter human and animal food. Adverse effects of acute exposure to DON include anorexia, diarrhea, and vomiting in experimental animals. Chronic DON exposure can lead to growth retardation and immunotoxic effects. Mice, commonly used in experimental studies for DON risk assessment, are incapable of vomiting but exhibit feed refusal and body weight suppression following exposure to the toxin.

Sex and age comparisons in C57BL6 mice to DON-induced anorexia were examined following acute i.p. and dietary exposure to the toxin. A bioassay for feed refusal was used to compare acute i.p. exposures of 1 and 5 mg/kg bw DON. Greater anorectic responses were seen in male than female mice and in aged mice (22 mos) than adult mice (3 mos). When effects of sex and age on food intake and body weight changes were compared after subchronic dietary exposure to 1, 2.5, and 10 ppm DON, males and aged mice were found again to be more sensitive than females and adults, respectively.

To identify contributing factors to sex and age dependent anorectic responses to DON, DON tissue clearance as well as plasma proinflammatory cytokine (IL-6, IL-1β, TNF-α) and satiety hormone (CCK, PYY) responses were measured. When acutely exposed to 1 mg/kg bw

DON i.p., male and aged mice displayed elevated DON tissue levels and delayed clearance in comparison with female and adult mice, respectively. When comparing sex differences, a significant increase in IL-6 plasma levels was observed in males while cholecystokinin (CCK) response was higher in females after acute DON exposure. In comparing adult and aged mice, acute DON exposure elicited higher proinflammatory cytokine (IL-6, IL-1β) and satiety hormone (CCK, PYY) responses in the plasma of the aged group compared with the adult group.

To further explain sex and age dependent anorectic DON responses, urinary and fecal excretion of the toxin were compared after acute i.p. exposure to 1 mg/kg bw DON. Males and aged mice were found to have slower urinary DON excretion in comparison with female and adult mice, respectively. In contrast, males and aged mice had greater excretion of the total DON dose in feces than female and adult mice, though fecal DON recovery accounted for a small percentage of the total DON dose recovered. Hepatic DON glucuronidation activity was found to be species specific, though sex and age differences were not consistently present. Additionally, renal DON glucuronidation activity by either mouse or mink microsomes was undetectable.

Identification of groups that show greater sensitivity to DON exposure and examination of factors related to these differences has the potential to provide important information to accurately assess the risk of DON consumption. The results presented in this dissertation indicate that anorectic responses to DON exposure have the potential to vary due to individual characteristics, including sex and age. Collectively, these findings suggest that sex and advanced life stage should be considered when formulating risk assessments for DON and other trichothecene mycotoxins.

## DEDICATION

To all of my past and present teachers and mentors

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## KEY TO ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| bw | Body weight |
| CART | Cocaine and amphetamine regulated transcript |
| CaSR | Calcium-sensing receptor |
| CCK | Cholecystokinin |
| COX 2 | Cyclooxygenase 2 |
| d | Day |
| DOM-1 | 9,12-diene deoxynivalenol; de-epoxy deoxynivalenol |
| DOM-GA | De-epoxy deoxynivalenol-glucuronic acid |
| DON | Deoxynivalenol |
| DON3GlcA | Deoxynivalenol-3-glucuronic acid |
| DON7GlcA | Deoxynivalenol-7-glucuronic acid |
| DON15GlcA | Deoxynivalenol-15-glucuronic acid |
| ds-RNA | Double-stranded RNA |
| EEC | Enteroendocrine cells |
| eIF2α | Eukaryotic initiation factor 2 α |
| ESI | Electrospray ionization |

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbent assay |
| EFSA | European Food Safety Authority |
| FDA | United States Food and Drug Administration |
| GI | Gastrointestinal |
| gp | Group |
| GPCR | G protein-coupled receptor |
| h | Hour |
| Hck | Hematopoietic cell kinase |
| hs | High sensitivity |
| ICR | Institute of Cancer Research |
| i.c.v. | Intracerebroventricular |
| IgA | Immunoglobulin A |
| IgAN | Immunoglobulin A nephropathy; IgA nephritis |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| i.p. | Intraperitoneal |
| IP3 | Inositol triphosphate |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinases |
| MC4R | Melanocortin 4 receptor |
| min | Minute |
| mos | Month |
| mRNA | Messenger ribonucleic acid |
| NPY | Neuropeptide Y |
| PBS | Phosphate buffered saline |
| PI | Post injection |
| PLC | Phospholipase C |
| PKR | Double-stranded RNA (ds-RNA)-associated protein kinase |
| POMC | Pro-opiomelanocortin |
| ppm | Part per million |
| PYY | Peptide YY |
| rep | Replicate |
| RSR | Ribotoxic stress response |
| SEM | Standard error of the mean |
| SULT | Sulfotransferase |

| | |
|---|---|
| TF | Transcription factor |
| TNF-α | Tumor necrosis factor-alpha |
| TRPA1 | Transient receptor potential ankyrin-1 |
| UDP-GA | Uridine diphosphate glucuronic acid |
| UGT | Uridine 5'-diphospho-glucuronosyltransferase |
| VGCC | Voltage gated calcium channel |
| v/v | Volume/volume |
| wk | Week |
| w/v | Weight/volume |

# INTRODUCTION

## 1. Background

Deoxynivalenol (DON, vomitoxin) is produced by *Fusarium graminearum* and is the most commonly occurring mycotoxin that contaminates cereal grains (Pestka, 2010b). DON is resistant to heat processing and can enter human and animal food. A recent study reported high levels of DON contamination in corn and wheat samples obtained from North America, suggesting that exposure to this mycotoxin is frequent (Rodrigues and Naehrer, 2012). Adverse effects of acute DON exposure include symptoms of gastrointestinal illness and innate immune system activation (Pestka *et al.*, 2004). Chronic DON exposure can lead to growth retardation and immune system dysregulation.

Our lab has proposed that two major danger signaling pathways mediate the toxicity of DON. These include the neuroendocrine system and the innate immune system (Table 1.1). Relative to the neuroendocrine response system, DON stimulates enteroendocrine cells (EEC) through calcium-sensing receptor (CaSR) and transient receptor potential ankyrin-1 (TRPA1) to mediate the release of satiety hormones including cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (Zhou and Pestka, 2015). In experimental animals, this outcome likely contributes to anorexia, emesis, and growth suppression. Regarding the innate immune system, DON induces proinflammatory cytokine release through activation of double-stranded RNA (ds-RNA)-activated protein kinase (PKR) and mitogen-activated protein kinases (MAPKs) in mononuclear phagocytes (Zhou *et al.*, 2003; Zhou *et al.*, 2014). Aberrant activation of the innate immune system could contribute to immune dysregulation, anorexia, and growth impairment.

Table 1.1 Proposed major danger signaling pathways that mediate DON toxicity.

| | **Neuroendocrine Response** | **Innate Immune Response** |
|---|---|---|
| **Sentinel Cells** | Enteroendocrine cells | Mononuclear phagocyte |
| **Sensors** | CaSR, TRPA1 | Ribosome/Double-stranded RNA protein kinase (PKR) |
| **Effectors** | Satiety hormones: e.g. CCK, PYY | Proinflammatory genes: e.g. IL-6, IL-1β, TNF-α |
| **Outcome** | Anorexia, impaired growth, emesis | Immune dysregulation, impaired growth, anorexia |

## 2. Rationale and Hypothesis

The long term goal of this research is to determine if factors such as sex and age should be taken into account during risk assessments of DON and other trichothecene and subsequent tolerable limits. The objective of this dissertation was to examine the effect of sex and age in susceptibility to DON-induced anorexia and identify factors that contribute to differences seen in sensitivity. Our guiding hypothesis is that sex and age increased susceptibility to DON-induced feed refusal and that is linked to aberrant cytokine and satiety hormone responses. Using a mouse model, our guiding hypothesis was addressed in three Specific Aims:

**Specific Aim 1:** Compare sex and age differences to DON-induced anorexia upon acute i.p. and dietary DON exposure.

**Specific Aim 2:** Relate induction of proinflammatory cytokine and satiety hormone responses to increased susceptibility to DON-induced anorexia.

**Specific Aim 3:** Relate tissue DON clearance and excretion to increased susceptibility to DON-induced anorexia.

## 3. Chapter Summaries

Chapter 1 is a literature review summarizing 1) previous research in sex-dependent responses to DON, 2) changes with aging that could lead to increased susceptibility of DON, and the 3) current knowledge of DON metabolism and clearance. First, previous research exploring sex-dependent weight suppression, food intake and IgA nephropathy with DON exposure is discussed. The anorexia of aging is also related to adverse effects of DON consumption. Second, the mechanisms and contributions of proinflammatory cytokine and satiety hormone responses to

DON-induced anorexia are described. The third portion of the literature review focuses on DON metabolism, distribution, and excretion.

Chapter 2 examines the effect of sex on DON-induced anorexia and relates these observed differences to toxin tissue clearance and induction of proinflammatory cytokine and satiety hormone responses. The major finding of this chapter was that male mice exhibited a predilection to DON-induced anorexia following i.p. and dietary exposure to the toxin. Acute i.p. exposed male mice exhibited slower tissue DON clearance and increased induction of plasma IL-6 in male mice compared with female mice. Data in this chapter have been published in *Toxins* and can be found at http://www.mdpi.com/2072-6651/7/8/2845.

Chapter 3 examines the effect of age on DON-induced anorexia and relates observed differences to toxin tissue clearance and induction of proinflammatory cytokine and satiety hormone responses. Results presented in this chapter illustrate the dramatic differences in anorectic responses to DON between aged mice (22 mos) and adult mice (3 mos). Substantial decreases in food consumption were observed in aged mice upon acute i.p. exposure to DON in comparison to adult mice. Additionally, tissue clearance was slower and proinflammatory cytokine and satiety hormone responses were greatly elevated in aged mice relative to their adult counterparts. Consistent with acute i.p. DON exposure, dietary exposure to the toxin also produced greater body weight suppression and reduction of food intake in aged mice than adult mice. Data in this chapter have been published in *Toxins* and can be found at http://www.mdpi.com/2072-6651/7/10/4199.

Chapter 4 compares DON excretion by sex and age and addresses if differences in the capacity to glucuronidate the toxin exist. Consistent with the findings in Chapters 2 and 3, male mice and aged mice were found to have slower urinary clearance of the toxin than female and

adult mice, respectively. While slower excretion was hypothesized to be the result of differential glucuronidation activity, neither DON glucuronide formation nor levels of the cofactor required for glucuronide formation (UDP-glucuronic acid) were affected by sex or age. Species specific glucuronidation by hepatic microsomes was also examined in this chapter. Urine and fecal samples from this study are currently undergoing LC-MS/MS analysis at a collaborating lab at the University of Vienna to further elucidate DON metabolite excretion profiles in urine and feces relative to sex and age. When data from collaborators is provided, a third paper will be prepared and submitted to a peer-reviewed journal.

In Chapter 5, the impact on animal and human health of differential sex and age responses to DON exposure are discussed. Important future directions of this research are also recommended.

# CHAPTER 1: Literature Review

## 1. Deoxynivalenol (DON)

Deoxynivalenol (DON, vomitoxin) is a type B trichothecene mycotoxin that is produced by *Fusarium graminearum* and that is frequently found to contaminate cereal crops including wheat, corn, and barely (Wu *et al.*, 2014a). DON is largely unaffected by food processing including cleaning, milling, and baking and is detectable in cereal based products worldwide (Trigo-Stockli, 2002; Rodrigues and Naehrer, 2012). Consumption of *Fusarium*-contaminated food has been linked to multiple human gastroenteritis outbreaks in Asian countries during the latter half of the 20$^{th}$ century (Bhat *et al.*, 1989; Pestka, 2010b). Turner et al. (2010) examined urinary DON levels in United Kingdom citizens and found that over 98% of individuals who consumed cereal products had detectable levels of DON in their urine (Turner *et al.*, 2008). This same study also found a positive correlation between amount of cereal products consumed and urinary DON levels.

DON's inhibition of protein synthesis is one way that the toxin is thought to elicit its adverse effects (Azcona-Olivera *et al.*, 1995). DON interferes with peptidyl transferase function on the ribosome, which consequently impairs initiation and elongation (Shifrin and Anderson, 1999). DON can also activate double-stranded RNA-associated protein kinase (PKR), which can phosphorylate eukaryotic initiation factor 2 α (eIF2α) (Zhou *et al.*, 2003). When phosphorylated, eIF2α inhibits translation. Our lab has additionally shown that DON promotes the degradation of 28s RNA, which could further interfere with ribosome function and translation (Li and Pestka, 2008).

Due to prevalence and safety issues associated with foodborne DON exposure, the U.S. Food and Drug Administration (FDA) has established advisory intake limits of 1 part per million (ppm) for humans (FDA, 2010). Additionally, the FDA recommends that DON consumption in cattle and poultry not exceed 10 ppm of toxin, with an advisory limit of 5ppm for all other animals. The European Food Safety Authority (EFSA) has set regulatory limits of DON consumption between 0.5 to 1.75 ppm for food products intended for adults and 0.2 ppm for food products intended for infants and young children (EFSA, 2013). EFSA also regulates DON levels in animal food, with limits ranging from 0.9 ppm (food products intended for swine) to 12 ppm (maize by-products intended for adult animals other than swine).

## 2. Sex and age are potential susceptibility factors for DON-induced anorexia

### *2.1 Sex-dependent feed intake and body weight suppression upon DON exposure*

Several investigations suggest that male animals are more sensitive than female animals to the adverse effects from DON consumption (Cote *et al.*, 1985; Greene *et al.*, 1994a; Greene *et al.*, 1994b; Rotter *et al.*, 1994). Rotter et al. (1994) found that male ICR mice on diets containing 2 to 8 ppm DON showed a greater suppression of weight gain and lower food consumption than females over a 14 d period compared to control animals (Rotter *et al.*, 1994). The same investigation also reported that the amount of DON consumed by body weight was lower in male mice (1.49 mg DON/kg bw/d) than in female mice (1.59 mg DON/kg bw/d), further supporting the contention that the response to this toxin differs by sex. A 2-year feeding analysis in B6C3F1 mice found that males fed DON diets consumed less food than females; however, this did not lead to a significant reduction in body weight (Iverson *et al.*, 1995).

Studies in swine have reported greater sensitivities in males to weight suppression and food intake than females (Cote *et al.*, 1985; Andretta *et al.*, 2012). In a study conducted by Cote et al. (1985) in swine, researchers found that castrated male swine (barrows) fed diets containing 3.1 ppm and 5.8 ppm DON had increased difficulty with consistent weight gain in comparison with female swine (Cote *et al.*, 1985). That investigation also reported that male swine did not resume normal growth rates after being removed from treatment diets, while female swine growth rates recovered. A meta-analysis found an average of weight reduction of 34% in DON-treated barrows, while female weights were only reduced by 2% (Andretta *et al.*, 2012). This investigation also reported that intake of DON-contaminated feed was suppressed in male swine compared with females, with reductions of 20% and 3%, respectively.

*2.2 Male predilection to DON-induced IgA nephropathy*

Investigations by our laboratory have also identified a male predilection to DON-induced IgA nephropathy (IgAN) and reported greater weight suppression in male mice than female mice (Greene *et al.*, 1994a; Greene *et al.*, 1994b; Greene *et al.*, 1995). In a study comparing IgAN by mouse strain and sex, it was found that B6FC1 males fed a diet containing 25 ppm DON for 8 wk lost 15% of their body weight while females of the strain and diet lost only 5% of their initial body weight (Greene *et al.*, 1994b). This investigation further found that male mice fed DON-free diet gained more weight (23% body weight increase) than female control mice (18% body weight increase).

*2.3 Anorexia of aging - DON as a potential contributing factor*

Previous studies have investigated the susceptibility of young animals to DON-induced anorexia (Cote *et al.*, 1985; Rotter *et al.*, 1994; Pestka and Amuzie, 2008; Andretta *et al.*, 2011;

Andretta *et al.*, 2012); however, the effects of this toxin on aged animals is largely unaddressed. Investigation of the adverse effects of DON in advanced life stage animals is important because approximately 25% and 30% of elderly men and women, respectively, are reported to be in an anorectic state (Thomas, 2009; Donini *et al.*, 2011; Martone *et al.*, 2013). This phenomenon of unintentional weight loss in advanced life stage has been termed the "anorexia of aging" and is a high predictor of morbidity and mortality in the elderly (Morley and Silver, 1988; Landi *et al.*, 2010). The etiology of anorexia is complex and thought to be caused by many factors including depression, increased susceptibility to illness, diet, and the burden of multiple medications (Chapman *et al.*, 2002; Mangoni and Jackson, 2004; Thomas, 2009). In a study of elderly Finnish men with depressive symptoms (65-84 years of age; n = 688), many reported gastrointestinal complaints over a 2 wk period including diarrhea (20%), stomach pains (37%), nausea (29%), vomiting (9%), and loss of appetite (21%) (Kivela *et al.*, 1988). Intriguingly, these symptoms are also present during DON intoxication. It is possible that elderly individuals who are already predisposed to gastrointestinal ailment might be more sensitive to the adverse effects of DON.

## 3. DON induction of proinflammatory cytokine and satiety hormone responses

### *3.1 DON induction of proinflammatory cytokines*

DON targets macrophages, T cells, and B cells in the immune system and the toxin's effects can be either immunostimulatory or immunosuppressive depending on dose and frequency of exposure (Pestka *et al.*, 2004). One potential cause of DON-induced anorexia is the induction of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, which have been

previously shown to cause sickness behavior in humans and experimental animals (Dantzer, 2001; Girardet *et al.*, 2011; Lebrun *et al.*, 2015). Exposure to low doses of DON stimulates the immune system by upregulating transcription and expression of proinflammatory genes in both *in vivo* and *in vitro* studies. DON induces IL-6, TNF-α, and COX-2 in macrophages (Sugita-Konishi and Pestka, 2001; Pestka, 2010a). In murine models, DON exposure also increases IL-6, IL-1β and TNF-α mRNA expression in spleen and liver tissue (Pestka and Amuzie, 2008). In contrast, exposure to high doses of DON causes apoptosis in macrophages, T cells and B cells in cell culture experiments (Uzarski *et al.*, 2003). This result is also reflected in mice exposed to 25 mg/kg bw DON which exhibit evident apoptosis in the spleen, thymus and Peyer's patches (Zhou *et al.*, 2000).

DON causes immunotoxic effects through a process known as the ribotoxic stress response (RSR) (Zhou *et al.*, 2014). This response is mediated through DON binding to the ribosome and inducing activation of double-stranded RNA (dsRNA)-associated protein kinase (PKR) and the Src kinase Hck (hematopoietic cell kinase), with subsequent downstream activation of mitogen-activated protein kinases (MAPKs). This results in the activation of transcription factors (TFs) and increased mRNA stability of immune system elements culminating in stimulation of the innate immune system (Pestka *et al.*, 2004). Prolonged MAPK activation by high DON concentrations can also lead to leukocyte apoptosis and immunosuppression.

DON exposure has previously been shown to induce IL-6, IL-1β and TNF-α proinflammatory cytokine expression in the female mouse model (Azcona-Olivera *et al.*, 1995; Pestka and Amuzie, 2008), but sex and age comparisons are lacking. However, aberrant induction of IgA by DON is greater in male mice than female mice and since IgAN is driven by

IL-6, males might produce more IL-6 in response to DON than females (Greene *et al.*, 1994a). While information on DON exposure and immune response with aging is lacking, prior investigations of the effect of lipopolysaccharide (LPS) in aged and adult mice have shown that the former exhibit a greater cytokine response to LPS than that of adults (Saito *et al.*, 2003; Huang *et al.*, 2008). Huang *et al.* (2008) found that plasma IL-6 levels of aged mice were 2- and 6.6-fold higher at 2 and 8 h post intracerebroventricular (i.c.v.) injection of 10 ng of LPS than adult mice (Huang *et al.*, 2008). Another study found that plasma levels of IL-1β and IL-6 were significantly increased in aged mice in comparison with adults at 6 h post exposure to 5 μg/g bw LPS administered by intraperitoneal (i.p.) injection (Saito *et al.*, 2003). Taken together these studies suggest that sex and age differences in proinflammatory cytokine response to DON might exist.

*3.2 DON induction of satiety hormones*

Our lab discovered that the gut satiety hormones peptide YY (PYY) and cholecystokinin (CCK) are induced in mice upon i.p. and oral DON exposure and both contribute to feed refusal caused by this mycotoxin (Flannery *et al.*, 2012; Wu *et al.*, 2014b). PYY is a 36-amino acid peptide that decreases food intake and is secreted by the L cells of the colon and ileum (Challis *et al.*, 2003). PYY is a potent anorexigenic hormone that has been shown to decrease mRNA expression of the orexigenic neuropeptide Y (NPY) and increase expression pro-opiomelanocortin (POMC), an anorexigenic peptide. CCK is a hormone that is secreted by I cells within the small intestine and also decreases food consumption (de Lartigue *et al.*, 2007). The satiety effect of CCK is short term and involves the upregulation of POMC and melanocortin 4 receptor (MC4R) expression (Fan *et al.*, 2004). The I and L cell types that release PYY and CCK, respectively, are known as enteroendocrine cells (EECs). While EEC account for 1% of

all gut epithelial cells, they still represent one of the largest endocrine organs in the body as a whole (Moran-Ramos *et al.*, 2012). This makes examination of these satiety hormones of particular interest when identifying contributing factors in increased sensitivity to DON-induced anorexia.

Recent research in our laboratory has employed a murine neuroendocrine tumor STC-1 cell line to investigate the mechanism of how DON induces the release of satiety hormones (Zhou and Pestka, 2015) (Figure 1.1). This research found that DON induces CCK secretion in SCT-1 cells by initially elevating intracellular calcium [$Ca^{2+}$] and activating the G protein-coupled receptor (GPCR) calcium sensing receptor (CaSR). Activation of CaSR was shown to then elicit the following serial downstream effects: (1) phospholipase C (PLC)-mediated activation of inositol triphosphate (IP3) receptor and mobilization of intracellular $Ca^{2+}$ stores, (2) activation of the transient receptor potential melastatin-5 ion channel (TRPM-5) $Na^{2+}$ channel and resultant L-type voltage gated calcium channel (VGCC)-facilitated $Ca^{2+}$ entry, and (3) amplification of extracellular $Ca^{2+}$ entry by transient receptor potential ankyrin-1 (TRPA1), which leads to $Ca^{2+}$ driven CCK exocytosis (Figure 1.1). While the STC-1 cell line was found to express PYY mRNA, the aforementioned study did not detect secretion of PYY under the same experimental conditions. Further research is needed to determine the mechanism through which DON induces PYY.

While previous studies have explored elevation of PYY and CCK in female animals following DON exposure, the effect of DON on satiety hormones in males and aged mice has yet to be addressed. Studies in humans have reported that females produce higher levels of PYY and CCK after food consumption (Burton-Freeman *et al.*, 2004; Kim *et al.*, 2005). Some

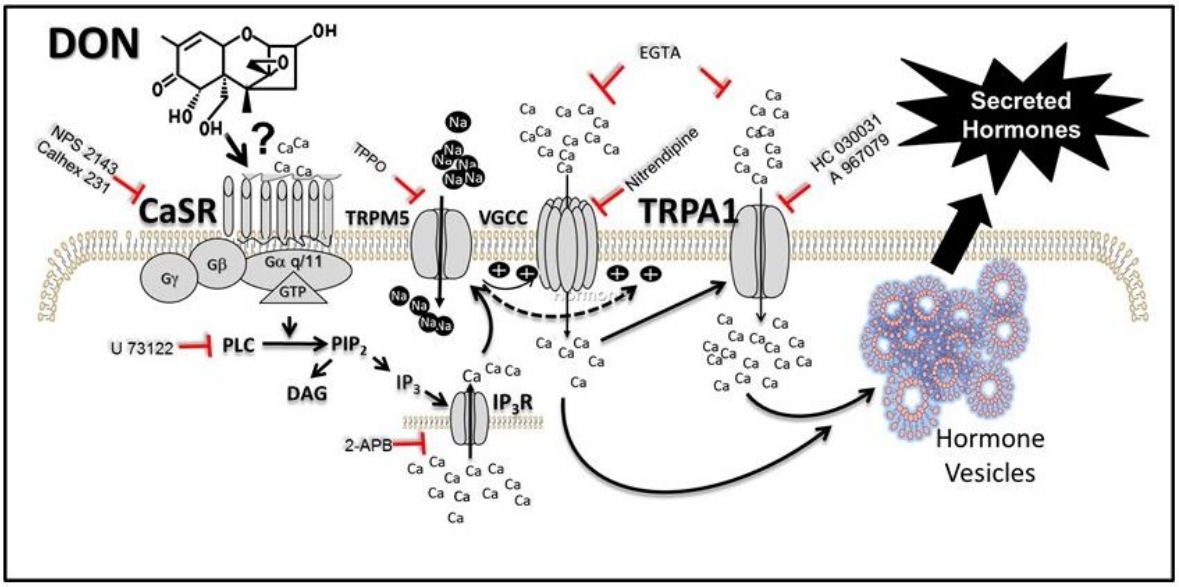


Figure 1.1 Putative model for DON-induced hormone secretion by the STC-1 EEC model. Previous research in our lab suggests that DON initiates $[Ca^{2+}]_i$ increase in STC-1 cells via CaSR-mediated pathway that involves the following serial events: (1) PLC-mediated activation of the IP3 receptor and mobilization of intracellular $Ca^{2+}$ (2) activation of the TRPM5 $Na^{2+}$ channel and resultant L-type VSCC-facilitated extracellular $Ca^{2+}$ entry, and (3) amplification of extracellular $Ca^{2+}$ entry by TRPA1 activation. The resultant elevation of $[Ca^{2+}]$ drives exocytosis of CCK, GLP-1, and potentially other hormones. Our lab proposes that DON hijacks these normal physiologic processes mediated by CaSR, thereby disrupting homeostasis and mediating hormone release. The blocking arrow symbol indicates targets of antagonists used to help identify pathway targets (Figure from Zhou *et al.*, 2015).

investigations have also reported higher levels of satiety hormones with aging (Sandstrom and El-Salhy, 2002; Moss *et al.*, 2012). When Stanström *et al.* (1998) compared the number of enteroendocrine cells responsible for PYY secretion in the intestinal epithelium of mice that were 3 and 24 months of age, they found that aged mice had significantly more of these cells (Sandstrom *et al.*, 1998). Another study in rats revealed that PYY-containing cells per colonic crypt increased with age (Sweet *et al.*, 1996). In an investigation comparing the effects of CCK in adult and aged mice, researchers found that aged mice showed increased sensitivity to CCK and that the action of CCK was prolonged in aged mice (Silver *et al.*, 1988). As PYY and CCK are induced by DON and tend to increase with age, exploring the combined effects of DON exposure and aging is of importance.

## 4. DON metabolism, distribution and excretion

### *4.1 DON metabolism*

Metabolism and excretion of DON is highly-species dependent (Wu *et al.*, 2010; Maresca, 2013). A de-epoxide derivative of DON (9,12-diene DON; de-epoxy DON; DOM-1) was the first metabolite identified and is the product of GI tract bacterial metabolism (Yoshizawa *et al.*, 1983; Worrell *et al.*, 1989). In poultry, bacterial isolates belonging to genera *Anaerofilum*, *Bacillus*, and *Collinsella* as well as the order Clostidiales have been identified as some organisms capable of transforming DON to DOM-1 (Yu *et al.*, 2010).

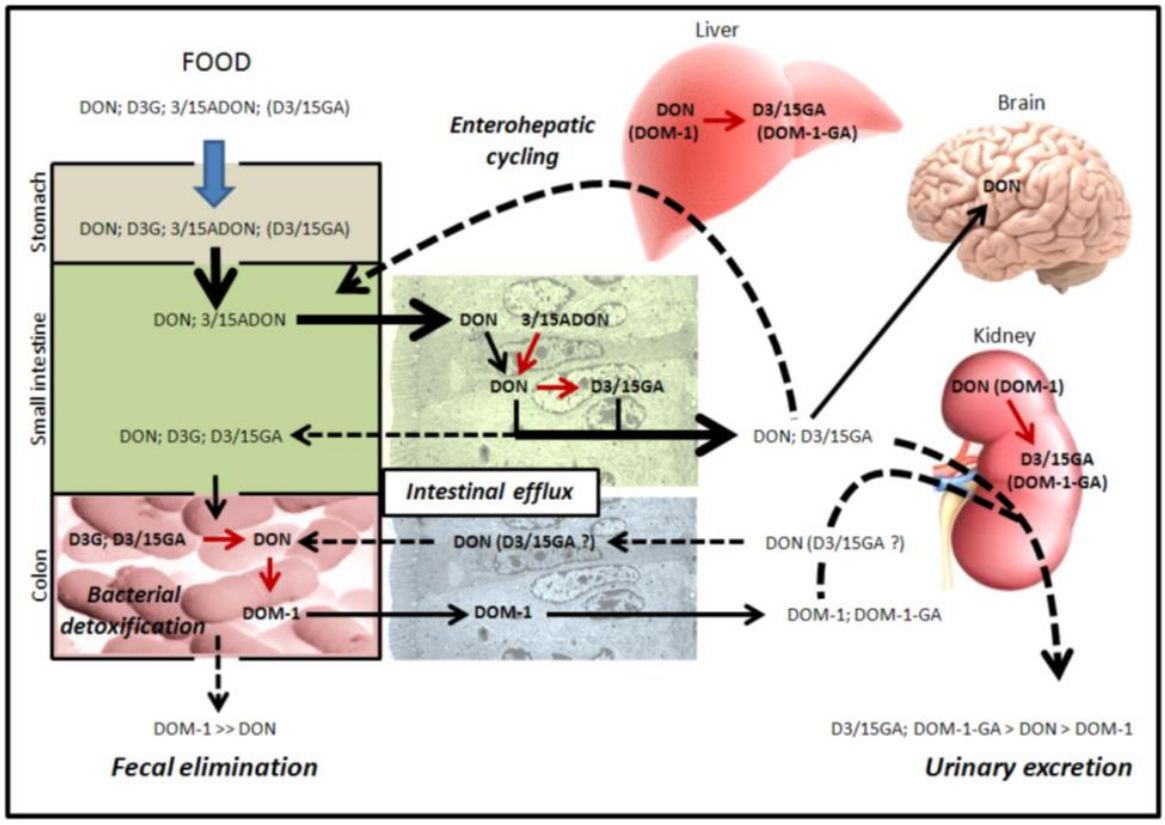


Figure 1.2 Diagram of the putative model of absorption, detoxification, and subsequent excretion pathways for DON in monogastric animals (i.e. rats, swine, humans). This diagram represents what is proposed to occur with oral exposure to the toxin. The addition of GA to native DON and metabolites represents the addition of glucuronic acid to the compound. Red arrows indicate transformation of DON and DON derivatives and dashed arrows indicate routes of elimination (Figure from Maresca 2013).

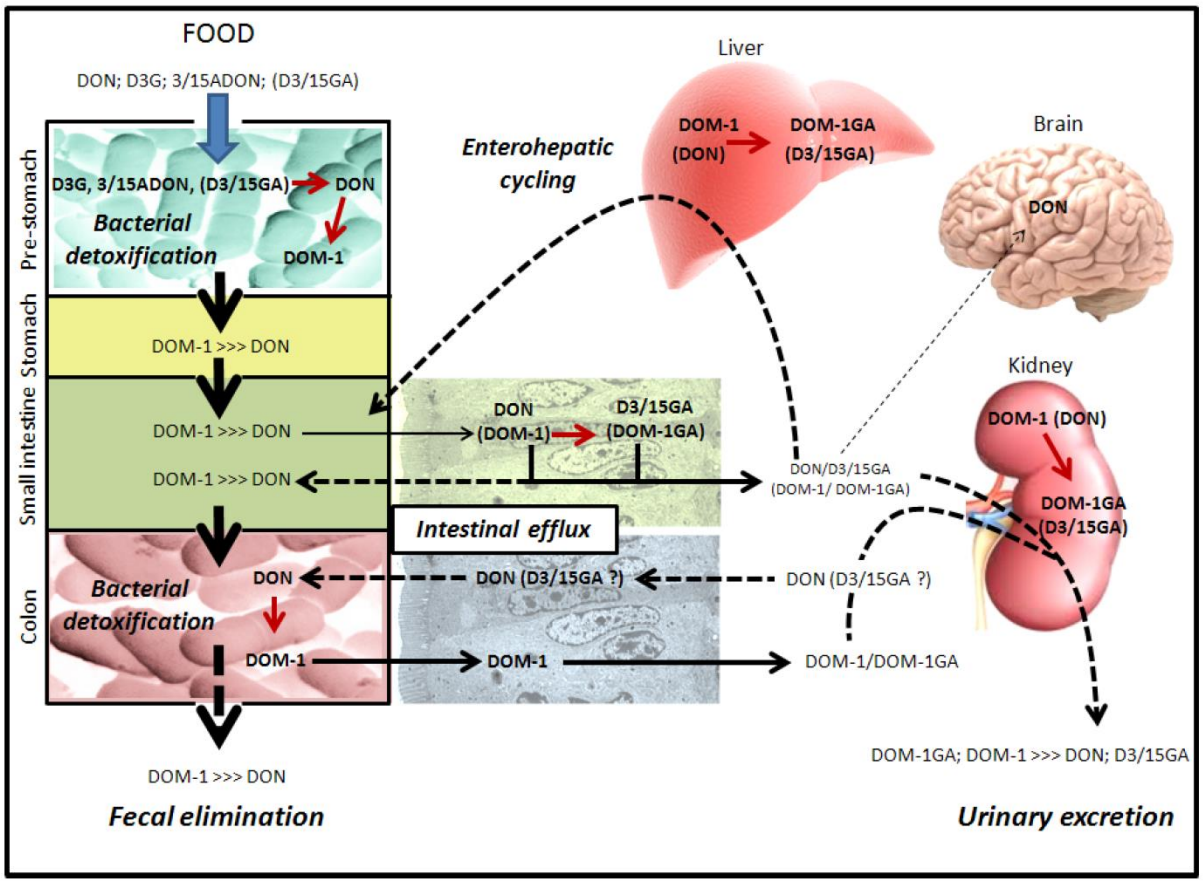


Figure 1.3 Diagram of the putative model of absorption, detoxification, and subsequent excretion pathways for DON in polygastric animals (i.e. cattle, poultry). This diagram represents what is proposed to occur with oral exposure to the toxin. The addition of GA to native DON and metabolites represents the addition of glucuronic acid to the compound. Red arrows indicate transformation of DON and DON derivatives and dashed arrows indicate routes of elimination (Figure from Maresca, 2013).

Polygastric animals (i.e. ruminants) and poultry have a high concentration of microflora present both before (i.e. rumen, croup) and after the small intestine, where DON is absorbed. Thus they are better able to produce DOM-1 than monogastric animals such as swine, rodents and humans (Smith, 1965; Avantaggiato *et al.*, 2004; Maresca, 2013) (Figures 1.2 and 1.3). DOM-1 formation is reported to occur at low or undetectable rates (Lattanzio *et al.*, 2011). One report found that only one of five human fecal samples incubated with DON was able to form DOM-1 (Gratz *et al.*, 2013).

Glucuronidation of DON is another widely studied form of metabolism. DON glucuronidation occurs through the action of uridine 5'-diphospho-glucuronosyltransferases (UGTs) and requires the co-factor uridine 5'-diphospho-glucuronic acid (UDP-GA). Sex and age differences have previously been identified in mRNA levels of UGTs in C57BL6 mice (Buckley and Klaassen, 2007; Buckley and Klaassen, 2009; Zhang *et al.*, 2010; Fu *et al.*, 2012). Formation of DON-3 glucuronic acid (DON3GlcA) is the dominant glucuronide formed by non-human animals with DON-7 glucuronic acid (DON7GlcA) being a minor glucuronide metabolite (Maul *et al.*, 2012; Maul *et al.*, 2015). An exception to this profile of glucuronide formation is the pig, which forms DON-15 glucuronic acid (DON15GlcA) as a minor metabolite instead of DON7GlcA. In comparison, humans produce DON15GlcA as the major glucuronide of DON with DON3GlcA being a minor glucuronide metabolite (Sarkanj *et al.*, 2013; Warth *et al.*, 2013; Maul *et al.*, 2015).

New reports published during this dissertation have shown that DON sulfonates can also result from DON metabolism. Wan et al (2014) found that 24% of the total DON dose was excreted as a yet uncharacterized DON sulfonate in rats orally exposed to 0.5 and 2.5 mg/kg bw to the toxin. In the same study, production of DON sulfonate metabolites were even higher in

poultry orally exposed to 2.5 mg/kg bw of the mycotoxin, with 89% of the total DON dose excreted as the tentatively named DON-3α-sulfate. Another paper reported that DON sulfonate metabolites were produced following oral exposure of rats to DON and 47% of the total DON dose was excreted as sulfonated DON metabolites (Schwartz-Zimmermann *et al.*, 2014a). How these DON and DOM sulfonates are formed is yet unknown, however they could be from metabolism by tissue sulfotransferases (Sults) or result from spontaneous formation in the intestinal tract (Schwartz-Zimmermann *et al.*, 2014b). Previous studies show evidence that mRNA levels of some Sults are higher in livers of female C57BL6 mice than males (Kocarek *et al.*, 2008; Fu *et al.*, 2012). While mRNA expression of Sults generally increases with age, Sult3a1 and Sult5a1 have been shown to decrease in the livers of both male and female C57BL6 mice with aging (Fu *et al.*, 2012).

*4.2 Enterohepatic cycling of conjugated compounds*

During enterohepatic cycling, xenobiotics or endogenous compounds are circulated from the liver to bile, then undergo reabsorption in the small intestine after which they travel back to the liver for systemic circulation (Roberts *et al.*, 2002; Gao *et al.*, 2014) (Figure 1.4). Conjugated compounds are excreted in the bile into the duodenum via the Sphincter of Oddi (Roberts *et al.*, 2002). Microflora of the GI tract are often involved in de-conjugation of the compounds which allows for their re-uptake in the small intestine, though some removal of transformation back to the native compound can occur through activity of the gut lumen cells (Ilett *et al.*, 1990; Sousa *et al.*, 2008). In the case of DON, bacteria producing β-glucuronidase (i.e. *Escherichia coli*) and arylsulfontransferase (e.g. *Streptococcus*) enzymes would be capable of transforming DON

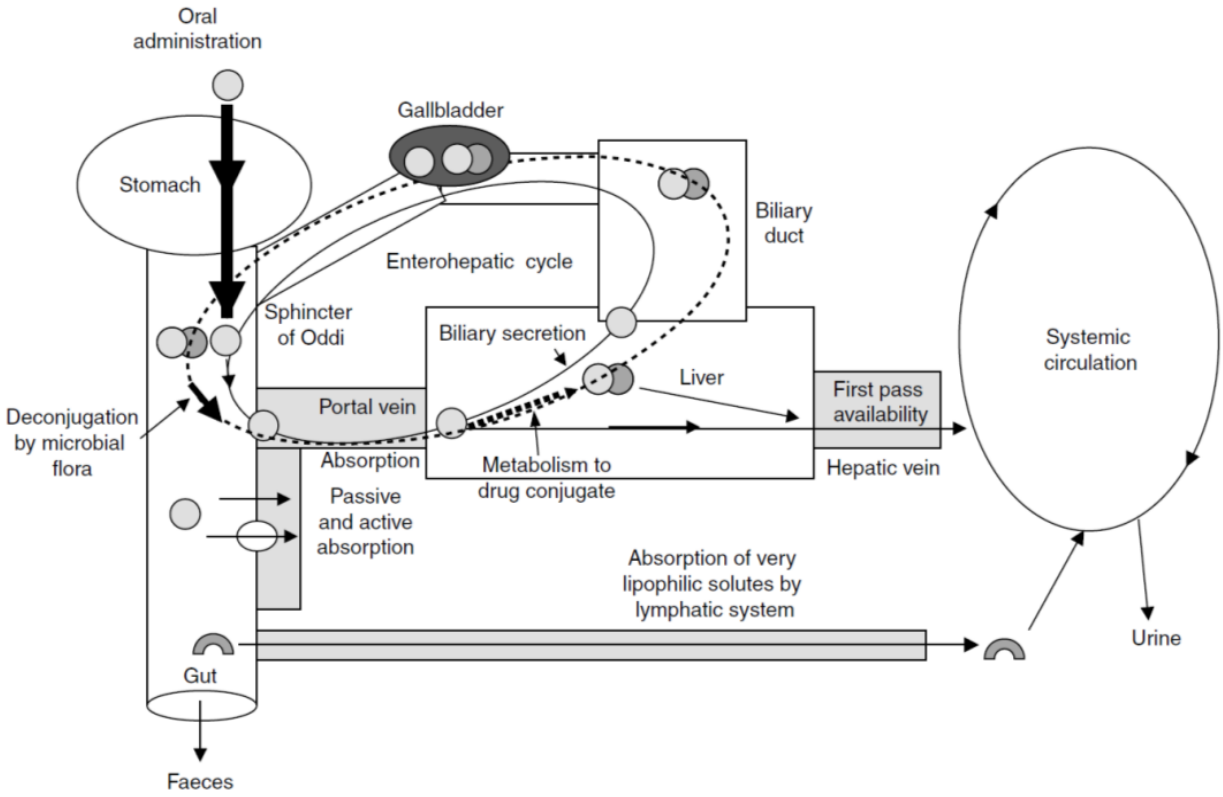


Figure 1.4 Enterohepatic recirculation. With oral administration, xenobiotics entering the GI tract may be absorbed into portal circulation. The compound is conjugated in the liver and can undergo biliary excretion to re-enter the GI tract via the sphincter of Oddi. In enterohepatic cycling, deconjugation by microflora can occur, making the compound susceptible to re-uptake (Figure from Roberts *et al.*, 2002).

glucuronides and sulfonates back to the native toxin, making it available for reuptkae (Ilett *et al.*, 1990; Maresca, 2013). This is important as enterohepatic recirculation can prolong the half-life of compounds. Coliform bacteria in the intestinal tract have been shown to increase with age, thus in individuals at advanced life stages enterohepatic cycling could be occurring at a greater rate (Gorbach *et al.*, 1967).

*4.3 DON distribution in animal tissues*

Previous research has shown that DON is rapidly distributed throughout the body in experimental animals (Azcona-Olivera *et al.*, 1995; Pestka *et al.*, 2008). In a study examining B6C3F1 mice orally exposed to 5 mg/kg and 25 mg/kg [$^{3}$H]DON, at 30 min or 1 h post exposure the following rank order of DON tissue distribution was observed: kidney>liver>small intestine>large intestine>spleen>Peyer's patches (Azcona-Olivera *et al.*, 1995). Plasma DON levels were higher than kidney toxin levels in mice exposed to 25 mg/kg at 30 min and 1 h post exposure, however all other values for plasma DON generally were ranked in between values reported for the liver and small intestine. In mice exposed to 5 mg/kg bw DON, toxin levels rapidly decreased in all tissues over a 24 h period. Interestingly, mice exposed to 25 mg/kg bw DON, however exhibited a second peak in toxin concentrations at 8 h post exposure in the kidney, liver, and small and large intestines after an initial decline in toxin levels in these organs from 30 min to 4 h following DON administration. This observation further supports the potential for DON to undergo enterohepatic circulation, especially at high toxin exposure levels.

Another investigation in B6C3F1 mice orally exposed to 5 mg/kg bw DON, compared toxin tissue distribution in weanling (3-4 wk) and adult (8-10 wk) mice using a competitive ELISA (Pestka and Amuzie, 2008). As in a study by Aconza-Oliveria *et al.* (1995), toxin levels were highest in the kidneys of both weanling and adult mice from 15 to 120 min post exposure.

In adult animals, tissue DON levels were relatively similar in the plasma and liver during exposure duration. In weanling mice, however, plasma toxin concentrations were slightly higher than liver DON concentrations at 15 and 30 min following DON exposure. Toxin concentrations in the spleens of both weanling and adult mice were lower than other mentioned tissues at all measured time points.

*4.4 DON excretion profiles in experimental animals*

In an early investigation examining DON excretion in male Wistar rats orally exposed to approximately 8-11 mg/kg of the toxin, researchers found that it was mostly excreted as DOM-1 in the urine and feces, accounting for 68% of the total recoverable dose (Yoshizawa *et al.*, 1983). However, total recovery of DON and the metabolite DOM-1 was low, with only 15% of the total DON dose recovered in the urine (9%) and feces (6%). Another study in male PVG rats orally exposed to 10 mg/kg bw radiolabeled [$^{14}$C]DON had substantially higher recovery with total excretion equaling 64% in feces, 25% in urine, and 0.15% expired into air (Lake *et al.*, 1987). The majority of the DON dose was recovered within 48 h after exposure, with 49% of the dose recovered in the feces and 24% in the urine. When samples were analyzed via HPLC to determine if DON was excreted as the native toxin or DOM-1, total recovery was lower with DON accounting for 30% of the radioactivity in excreta and DOM-1 for 13%.

Previous research using radiolabeled DON found high recoveries of the radioactive dose in excreta of animals, however when analysis of specific metabolites was under taken, recovery rates of the total DON dose were much lower. For example, Nagl et al (2012) was only able to recover 28% of the DON dose orally administered to rats when urine and feces were analyzed for DON, DOM-1, and DONGlcA, suggesting that other metabolites existed (Nagl *et al.*, 2012). Wan et al (2014) reported that 24% of the total DON dose was excreted as a yet uncharacterized

DON sulfonate in rats orally exposed to 0.5 and 2.5 mg/kg bw DON (Wan *et al.*, 2014). Importantly, this study also reported sex differences in urinary excretion of DON in rats and found that male rats orally exposed to 0.5 and 2.5 mg/kg bw DON excreted 30% and 18% less of the total cumulative dose in urine, respectively, than female rats. In the same report, production DON sulfonate metabolite was even higher in poultry orally exposed to 2.5 mg/kg bw DON, with 89% of the total DON dose excreted as the tentatively named DON-3α-sulfate.

Another recent report of DON sulfonate metabolites produced by rats after oral exposure, found recovery rates of the total DON dose boosted from 28% to 75% when DON and DOM sulfonates were included in the analysis (Schwartz-Zimmermann *et al.*, 2014a). It was found that DON and DOM sulfonates accounted for 49% of recovered metabolites in feces, with DON sulfonate 2 (DONS 2) and DOM sulfonate 2 (DOMS 2) being the major metabolites formed.

## 5. Significance

Greater recognition of the critical importance of sex- and age-dependent responses to xenobiotics and the disparity of information available for differential susceptibilities of many compounds has been recognized as an important gap of knowledge in pharmacological and toxicological research. This dissertation details vital differences in sensitivity to adverse effects of toxin exposure that vary by sex and advanced life stage. Research presented herein supports the contention that of sex- and life stage-dependent responses to xenobiotic exposure are critical considerations for future risk assessment of this toxin.

## CHAPTER 2: Murine Anorectic Response to Deoxynivalenol (Vomitoxin) is Sex-Dependent

Data in this chapter has been published in Clark, E.S., Flannery, B.M., and Pestka, J.J. (2015). Murine anorectic response to deoxynivalenol (vomitoxin) is sex-dependent. *Toxins* 7, 2845-2859 (dio: 10.3390/toxins7082845).

### Abstract

Deoxynivalenol (DON, vomitoxin), a common trichothecene mycotoxin found in cereal foods, dysregulates immune function and maintenance of energy balance. The purpose of this study was to determine if DON's anorectic responses in mice differ by sex. A bioassay for feed refusal, previously developed by our lab, was used to compare acute i.p. exposures of 1 and 5 mg/kg bw DON in C57BL6 mice. Greater anorectic responses to DON were seen in male than female mice. Male mice had higher organ and plasma concentrations of DON upon acute exposure than their female counterparts. A significant increase in IL-6 plasma levels was also observed in males while cholecystokinin response was higher in females. When effects of sex on food intake and body weight changes were compared after subchronic dietary exposure to 1, 2.5, and 10 ppm DON, males were found again to be more sensitive. Demonstration of male predilection to DON-induced changes in food intake and weight gain might an important consideration in future risk assessment of DON and other trichothecenes.

## 1. Introduction

Deoxynivalenol (DON, vomitoxin) is a trichothecene mycotoxin produced by the fungus *Fusarium graninearum* that contaminates corn, wheat, and barley (Pestka, 2010b). DON is highly resistant to heat processing and can enter human and animal food. A recent study reported high levels of DON contamination in corn (76%) and wheat (79%) samples obtained from North America, suggesting that exposure to this mycotoxin is frequent (Rodrigues and Naehrer, 2012). In experimental animals, adverse effects of acute exposure include symptoms of gastrointestinal illness while chronic exposure can lead to growth retardation and immunotoxic effects (Trenholm *et al.*, 1984; Iverson *et al.*, 1995; Amuzie and Pestka, 2010; Voss, 2010). In species capable of emesis (e.g. pig, mink), DON rapidly induces vomiting (Forsyth *et al.*, 1977; Wu *et al.*, 2013). Rodents however are incapable of vomiting and instead exhibit feed refusal following exposure to this toxin (Forsell *et al.*, 1986; Flannery *et al.*, 2011).

Previous investigations suggest that male animals are more sensitive than female animals to the adverse effects from DON consumption (Cote *et al.*, 1985; Greene *et al.*, 1994a; Greene *et al.*, 1994b; Rotter *et al.*, 1994). Studies in swine have reported greater sensitivities in males to weight suppression and food intake than females (Cote *et al.*, 1985; Andretta *et al.*, 2012). An investigation conducted in ICR mice found that males fed DON containing diets showed greater growth depression and lower food intake than female mice (Rotter *et al.*, 1994). The same study also reported that the amount of DON consumed by body weight was lower in male mice (1.49 mg DON/kg bw/d) than in female mice (1.59 mg DON/kg bw/d) further supporting the contention that the response to this toxin differs by sex. A 2-year feeding study in B6C3F1 mice found that males fed DON diets consumed less food than females, though this did not lead to a significant reduction in body weight (Iverson *et al.*, 1995). Finally, prior investigations by our laboratory

identifying a male predilection to DON induced IgA nephropathy also reported greater weight suppression in male mice than female mice (Greene *et al.,* 1994a; Greene *et al.,* 1994b; Greene *et al.*, 1995).

DON-induced feed refusal has been linked to the induction of proinflammatory cytokines (i.e. IL-1β, IL-6, and TNF-α) which are known to have anorectic actions in animals and humans (Dantzer, 2001; Girardet *et al.*, 2011; Lebrun *et al.*, 2015). DON exposure has previously been shown to induce these proinflammatory cytokine expression in the female mouse model (Azcona-Olivera *et al.*, 1995; Pestka and Amuzie, 2008). Since immune dysregulation of IgA is greater in male mice than female mice, comparison of sex differences in these cytokines is also of interest.

Elevation of the satiety hormones cholecystokinin (CCK) and peptide YY (PYY) have also been observed upon DON exposure (Flannery *et al.*, 2012; Wu *et al.*, 2014b). CCK is a hormone that is secreted by I cells within the small intestine and it increases the expression of anorexinigenic peptides including cocaine and amphetamine regulated transcript (CART) (de Lartigue *et al.*, 2007). PYY is a 36-amino acid peptide that also decreases food intake by increasing the expression of anorexigenic peptides and it is secreted by the L cells of the colon and ileum (Challis *et al.*, 2003). While previous studies have explored elevated levels of these hormones in female animals following DON exposure, the effect of DON on satiety hormones in males has yet to be addressed.

The aims of this study were to evaluate and characterize murine sex differences in food refusal response after acute and dietary DON exposure. Study 1 addressed sex differences in feed refusal response to acute i.p. DON exposure. Study 2 compared DON tissue concentrations, as well as proinflammatory cytokine and satiety hormone responses to acute DON exposure in males and females. Study 3 compared sex differences in food intake and body weight suppression upon exposure to dietary DON. The results indicate that male mice were more sensitive than female

mice to acute i.p. and dietary DON exposure and that these differences correspond to slower toxin organ clearance and an increased IL-6 response with acute i.p. exposure.

## 2. Results and Discussion

### *2.1 Study 1*

#### *2.1.1 Feed refusal upon acute DON exposure is greater in males than females*

The effects of acute i.p. exposure to DON on food intake were compared in male and female mice over a period of 36 h (Figure 2.1). Male mice treated with 1 mg/kg bw DON ate 45% less food then control males at 6 h post-injection (PI) (Figure 2.2). After 6 h, male mice showed recovery with cumulative food intake nearly reaching that of controls 36 h PI. In contrast, female mice treated with 1 mg/kg bw DON ate 24% less food than female control mice at 6 h PI and began to show recovery in food intake at 5 h PI. Male mice treated with 5 mg/kg bw exhibited the greatest difference in food intake from the control males between 7 and 12 h PI, consuming 7% of food intake compared with control males. Food intake in males exposed to 5 mg/kg bw DON started to increase after 12 h PI, however, at 36 h PI males had consumed only 82% of what control males had eaten. Conversely, female mice treated with 5 mg/kg bw DON started to show recovery in food intake at 7 h after DON treatment and had eaten 33% of that by control females. At the final time point of 36 h PI, food intake of female mice treated with 5 mg/kg bw DON had almost completely recovered with the animals having cumulatively consumed 95% of the food intake of control females.

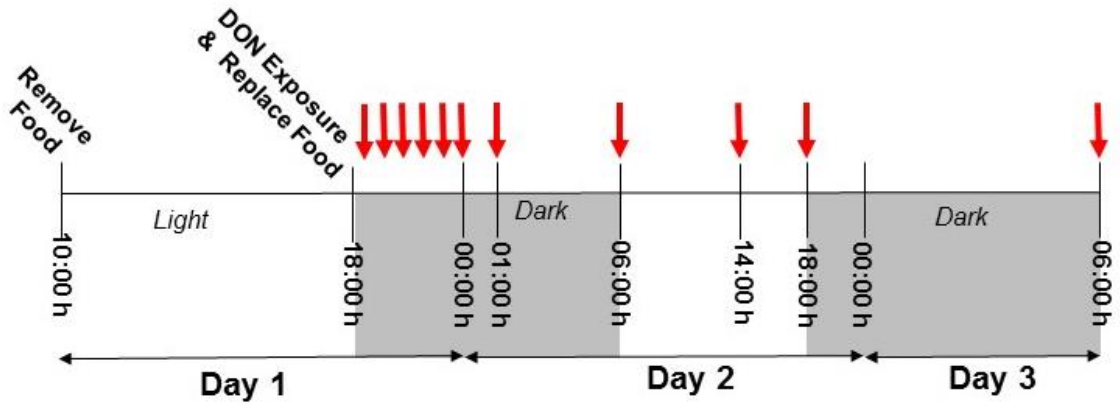


Figure 2.1 Study 1 experimental design. On day 1, mice were fasted from 10:00 to 18:00 h then exposed to DON treatments or vehicle control. Food was immediately replaced following exposure and food measurements were recorded hourly from 1 to 7 h PI, and at 12, 20, 24, and 36 h PI as indicated by arrows.

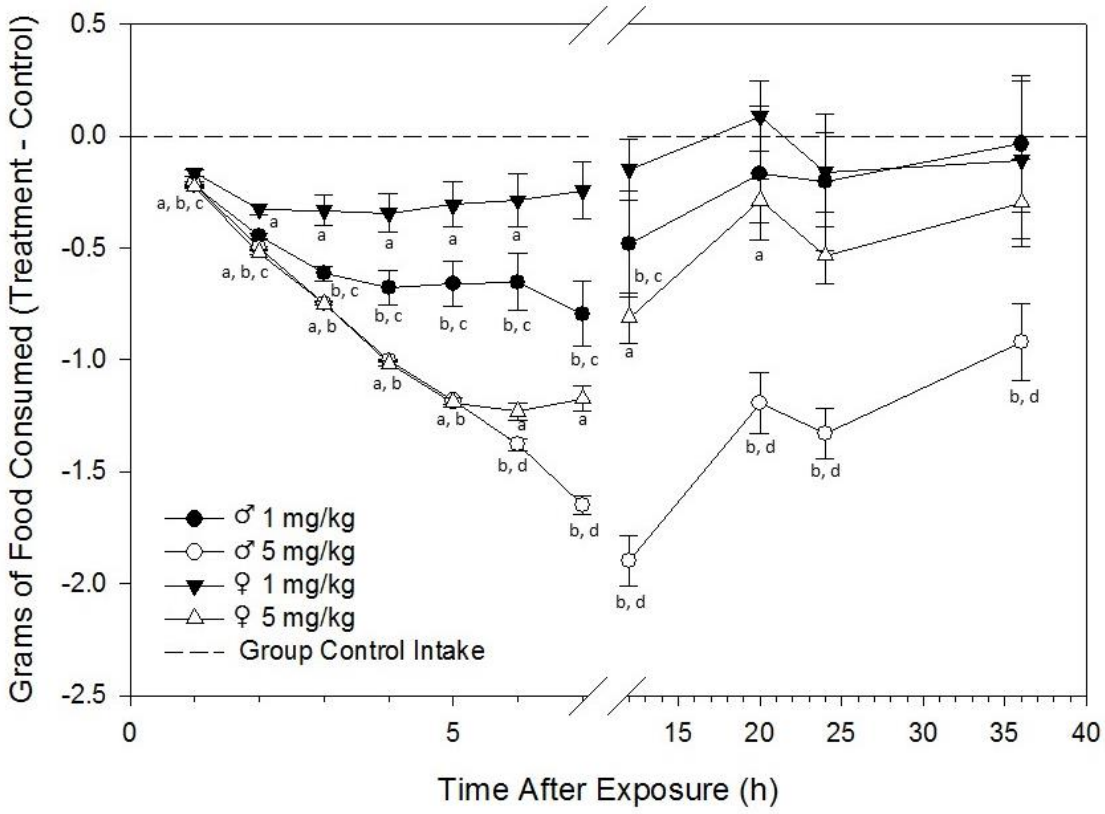


Figure 2.2 Male mice are more sensitive to DON-induced feed refusal than females following acute i.p. exposure. Food intake is cumulative at each time point and was normalized to group control average. Data are mean ± SEM (n = 10/gp). Statistically significance differences are indicated as follows: a = DON dosed female different from female control; b = DON dosed male different from male control; c = male different from female at 1 mg/kg bw DON; and d = male different from female at 5 mg/kg bw DON ($p < 0.05$).

While this is the first comparison of murine sex differences in DON-induced anorectic effects, acute feed refusal has been previously described in B6C3F1 female mice. Flannery et. al. (Flannery *et al.*, 2011; Flannery *et al.*, 2012) observed that by 4 h PI food intake had recovered in female B6C3F1 mice treated with 1 mg/kg bw DON i.p.. In this study, we found that C57BL6 females at the same time point and dose continued to show feed refusal, consuming 37% food than control females. These findings are consistent with reports of greater sensitivity of C57BL6 females to body weight reduction and mortality with DON exposure in comparison to B6C3F1 females (Greene *et al.*, 1994b).

### *2.2 Study 2*

#### *2.2.1 DON organ concentrations are higher in male mice after acute DON exposure*

Tissues were analyzed for DON following acute exposure to 1 mg/kg bw at 1, 2, and 4 h PI. DON concentrations were represented as DON equivalents as the ELISA used to quantify DON is 100% cross-reactive with the DON metabolite deoxynivalenol-3-glucuronide (DON3GlcA) (Figure 2.3). At 1 h PI, kidney, liver, and heart DON equivalent concentrations were higher in male mice than female mice (Table 2.1). Males had the highest concentration in kidney followed by the plasma. Female mice at this time point had the highest concentration of DON equivalents in the plasma, followed by the kidney. The rank order of DON equivalent concentrations in all other organs for males and females was: liver > heart > spleen > brain. Similar tissue distribution patterns have previously been reported in B6C3F1 mice and pigs (Prelusky and Trenholm, 1991; Pestka *et al.*, 2008). High concentrations of DON equivalents in the kidney of male mice at 1 h after DON treatment could indicate that male mice are excreting the toxin more slowly than female mice.

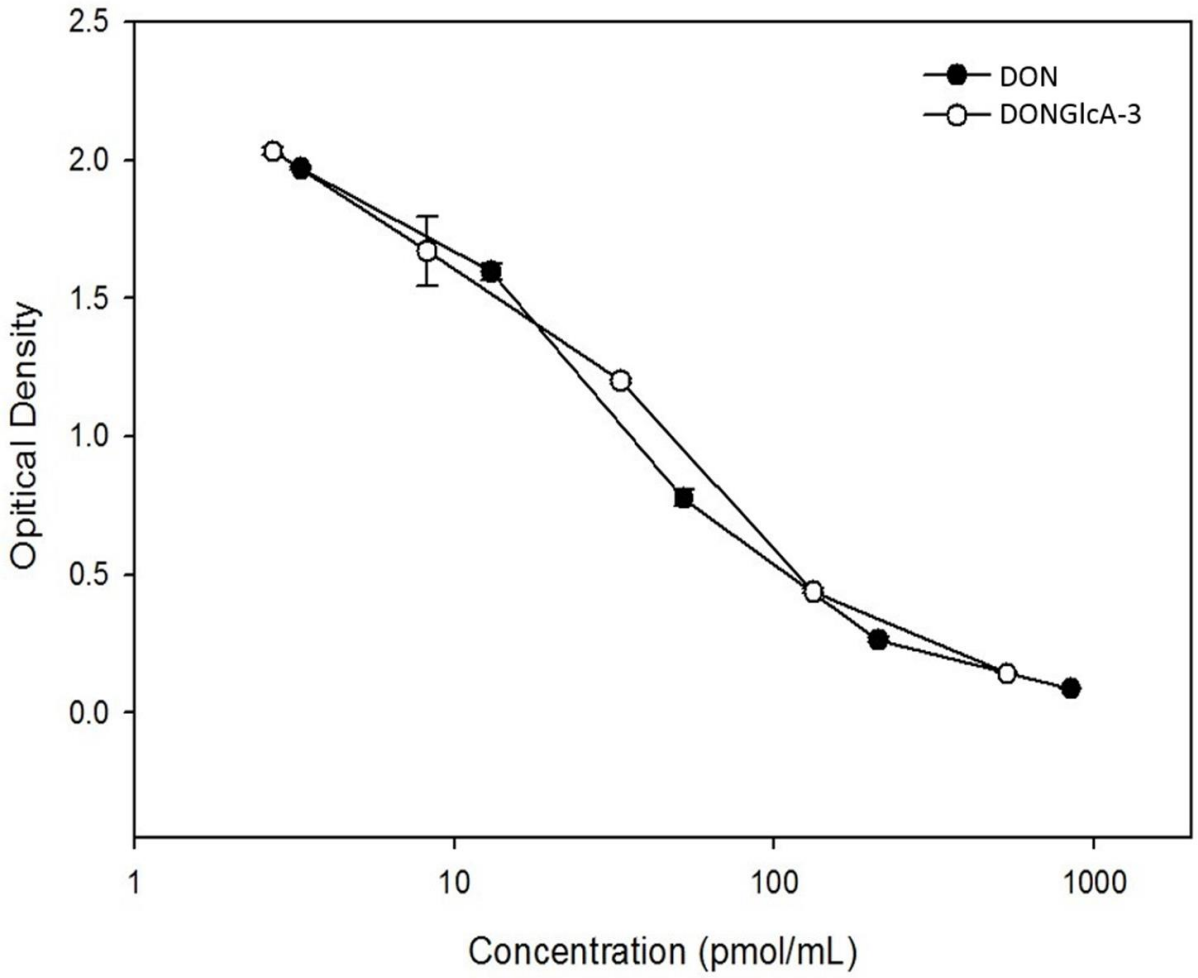


Figure 2.3 DON3GlcA is cross-reactive with DON in Veratox high sensitivity (hs) ELISA. The DON Veratox hs ELISA is a direct competitive ELISA using Four Parameter Logistic curve-fitting analysis. The x-axis is logistic and the y-axis is linear. Data are mean ± SEM (n=2 rep). DON3GlcA is the major metabolite formed in the rat liver. Cross reactivity with other potential glucuronides was not determined. Since we did not measure ratio of active vs. conjugated DON, results in dissertation are reported as DON equivalents.

Table 2.1 DON equivalent concentrations are higher in male mice at 1 and 4 hr post-acute i.p. exposure to 1 mg/kg bw DON.

| | DON Equivalents (nmol/g) | | | |
|---|---|---|---|---|
| | 1 h | | 4h | |
| **Organ** | ♂ | ♀ | ♂ | ♀ |
| **Kidney** | 3.85 ± 0.3* | 2.21 ± 0.2 | 0.30 ± 0.03* | 0.17 ± 0.01 |
| **Liver** | 2.60 ± 0.2 $^{p=0.09}$ | 1.86 ± 0.3 | 0.36 ± 0.1* | 0.14 ± 0.02 |
| **Plasma** | 2.72 ± 0.2 | 2.33 ± 0.2 | 0.20 ± 0.01* | 0.12 ± 0.01 |
| **Heart** | 2.24 ± 0.1* | 1.60 ± 0.2 | 0.19 ± 0.01* | 0.11 ± 0.01 |
| **Spleen** | 1.44 ± 0.1 | 1.16 ± 0.2 | 0.17 ± 0.01* | 0.10 ± 0.01 |
| **Brain** | 0.61 ± 0.01 | 0.64 ± 0.04 | 0.33 ± 0.02 | 0.30 ± 0.05 |

DON equivalent concentrations are nmol/g. DON is reported as DON equivalents as the ELISA was found to be completely cross reactive with DON3GlcA. Control animals did not have detectable levels of DON (data not shown). Data are mean ± SEM (n = 5-6/gp). Asterisk indicates statistical significance from female at time point ($p < 0.05$).

No differences in DON equivalent organ concentrations between males and females were observed at 2 h PI (data not shown). However, DON equivalent concentrations were significantly higher in males than females in all organs and plasma at 4 h post exposure, with the exception of the brain (Table 2.1). Interestingly, the brain showed the lowest change in DON equivalents from 1 to 4 h, with the concentration remaining to be approximately 50% of the 1 h levels measured in both male and female mice while all other organs had decreased DON levels from 7 to 14% of the 1 h measurement. Higher toxin levels remaining in the tissues of male mice could be a contributing factor to the increased feed refusal seen in males.

Overall, these data suggest that males may differ from females in absorption and/or clearance of DON. One possible metabolic difference is that males have a reduced capacity to glucuronidate, thus excrete the toxin. Sex differences have previously been identified in mRNA levels of UDP-glucuronosyltransferases (UGTs), the family of enzymes responsible for DON glucuronidation (Buckley and Klaassen, 2007; Buckley and Klaassen, 2009). Analyzing possible sex differences in DON metabolism will be an important objective for future studies.

*2.2.2 Plasma IL-6 is higher in male mice than female mice upon acute DON exposure*

When the effects of acute exposure to 1 mg/kg bw DON on plasma levels of proinflamatory cytokines were determined, DON treated males and females both had significantly higher IL-6 plasma levels than their respective group control at 1 and 2 h PI (Figure 2.4). Plasma IL-6 concentrations were elevated 2.25 fold higher at 2 h after DON treatment in male mice when compared with female mice. The high increase in this anorectic cytokine could be another factor to male sensitivity to acute DON-induced anorexia.

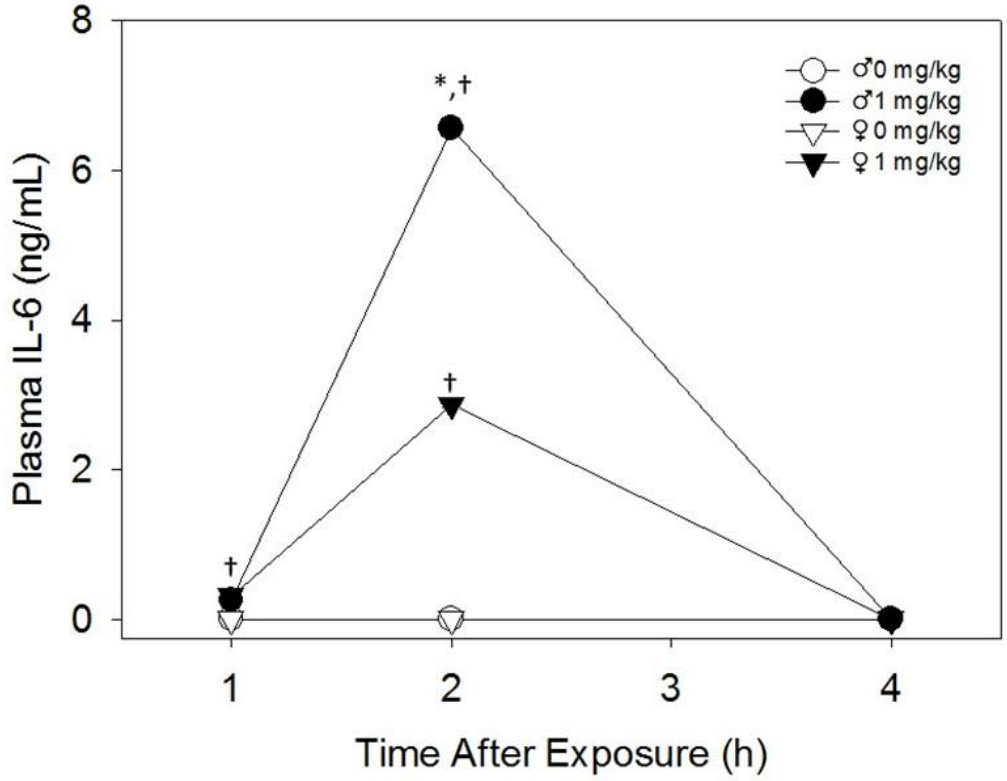


Figure 2.4 Male mice exhibit higher IL-6 plasma levels at 2 h post acute i.p. exposure to 1 mg/kg bw DON. Data are mean ± SEM (n = 4/rep). Asterisk indicates statistical significance from female at time point and dagger indicates significance from group control at time point ($p < 0.05$).

Plasma TNF-α and IL-1β were not induced by DON (data not shown). These findings are consistent with previous studies reporting very low levels of IL-1β and no increase in TNF-α plasma levels with treatments below 12.5 mg/kg bw DON (Islam and Pestka, 2003; Amuzie *et al.*, 2009).

*2.2.3 Plasma CCK and PYY are increased in both female and male mice with DON exposure*

DON treatment increased plasma CCK and PYY concentrations in both male and female mice in comparison to control animals at 1 and 2 h PI, returning to baseline levels by 4 h PI (Figure 2.5). Relative levels of gut satiety hormones in DON-treated female mice tended to be slightly higher than male mice, but only plasma CCK at 2 h was significantly higher in females. Thus, increased sensitivity of male mice to DON-induced feed refusal could not be explained by differences in either of these two satiety hormones.

*2.3 Study 3*

*2.3.1 Initial food intake is suppressed in male mice exposed to 10 ppm dietary DON*

When food intake was measured during Study 3, DON-fed male mice exhibited significant differences in the amount of food consumed at 1 and 2 d (Table 2.2). At these two time points there was significant negative correlation between decreasing food consumption with increasing DON treatments. At 2 d of treatment, males on diets containing 2.5 and 10 ppm DON were eating 15% and 25% less respectively than control animals. Translating such a decrease to a 2000 kcal diet would correspond to a reduction of eating 1700 and 1500 calories, respectively. In comparison, the females did not display a significant suppression in food intake. While female mice showed a trend of decreasing food consumption on d 1 of treatment, the correlation was only approaching statistical

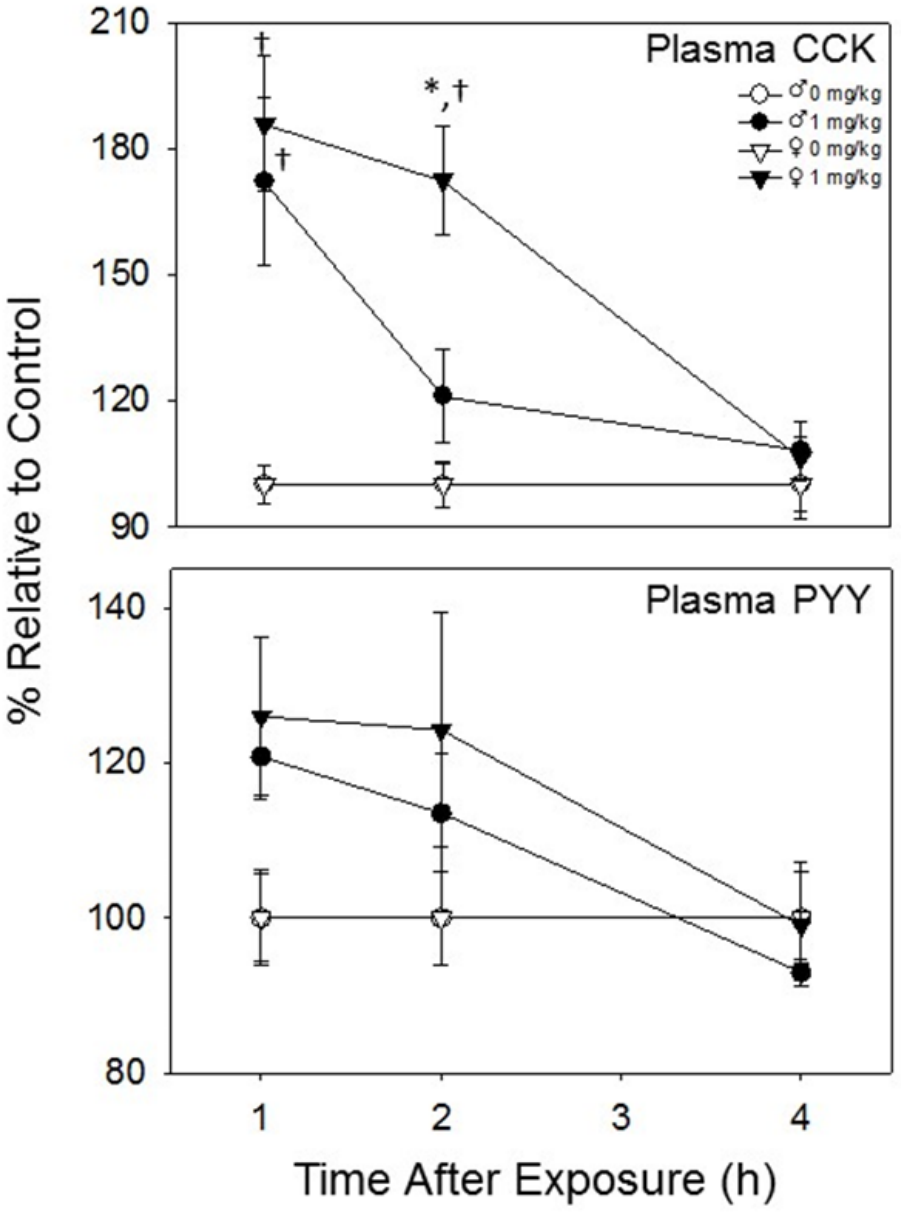


Figure 2.5 DON induces plasma CCK and PYY elevation. All values are reported as percent of the group control as control females had higher baseline levels of both gut satiety hormones. Data are mean ± SEM (n = 5-6/gp). Asterisk indicates statistical significance from male at time point and dagger indicates significance from group control at time point ($p < 0.05$).

Table 2.2 Food consumption is decreased in male mice fed DON containing diets.

| Group | % Control Food Intake 0-24 h | % Control Food Intake 24-48 h |
|---|---|---|
| ♂ **1 ppm** | 99.5 ± 3.8* | 95.2 ± 7.2* |
| ♂ **2.5 ppm** | 85.7 ± 7.3* | 85.0 ± 4.9* |
| ♂ **10 ppm** | 56.9 ± 10.2* | 74.9 ± 5.6* |
| ♀ **1 ppm** | 100.7 ± 4.2 | 95.6 ± 4.6 |
| ♀ **2.5 ppm** | 99.5 ± 8.1 | 101.4 ± 5.9 |
| ♀ **10 ppm** | 84.0 ± 7.8 | 92.3 ± 8.0 |

Values are percent of group control food intake. Data are mean ± SEM (n = 6/group). Asterisk indicates statistical significance in correlations between increasing dose and decreasing food intake at time point ($p < 0.05$). Correlation coefficients were: (males 0-24 h; $r = -0.731$; $p = 0.00009$), (females 0-24 h; $r = -.384$, $p = 0.06$), (males 24-48 h; $r = -0.495$; $p = 0.01$), and (females 24-48 h; $r = -0.16$; $p = 0.46$).

significance. After 2 d, mice fed diets containing DON progressively began to shred the food pellets precluding accurate food recovery after this time point.

The greater suppression of food intake with dietary DON exposure in male mice compared with females was consistent with the observation of an increased anorectic effect in males versus females in our acute i.p. DON exposure Study 1. These results also correspond to sex differences in food intake with DON exposure that have previously been reported (Rotter *et al.*, 1994; Iverson *et al.*, 1995; Andretta *et al.*, 2012). Studies in ICR and B6C3F1 mice reported a greater decrease in male food intake with dietary DON exposure when compared with females (Rotter *et al.*, 1994; Iverson *et al.*, 1995). A meta-analysis performed in swine research also reported that food intake with DON exposure was suppressed in males compared females, with reductions of 20% and 3% respectively (Andretta *et al.*, 2012).

*2.3.2 Dietary DON exposure causes greater suppression of weight gain in male mice*

When the effects of dietary exposure to DON at 0, 1, 2.5, and 10 ppm on body weight were compared over 17 d, male mice showed a significant decrease in body weight while female mice did not (Figure 2.6; Table 2.3). Males fed 10 ppm DON exhibited significantly suppressed weight gain in comparison to control males and females fed 10 ppm DON diet beginning at d 3 of treatment and they maintained this suppression until the end of the experiment. At the termination of the study, males fed with 10 ppm DON diet weighed 17% less than group controls. After 17 d of exposure to the diet containing 10 ppm DON, female mice exhibited only a 6% depression of body weight gain compared with group controls. Males on diets containing 1 and 2.5 ppm DON weighed 7% less than group controls at the end of the study, while females on these diets weighed only weighed 2% less than group controls. Adaptation to the toxin and increased metabolic capacity of

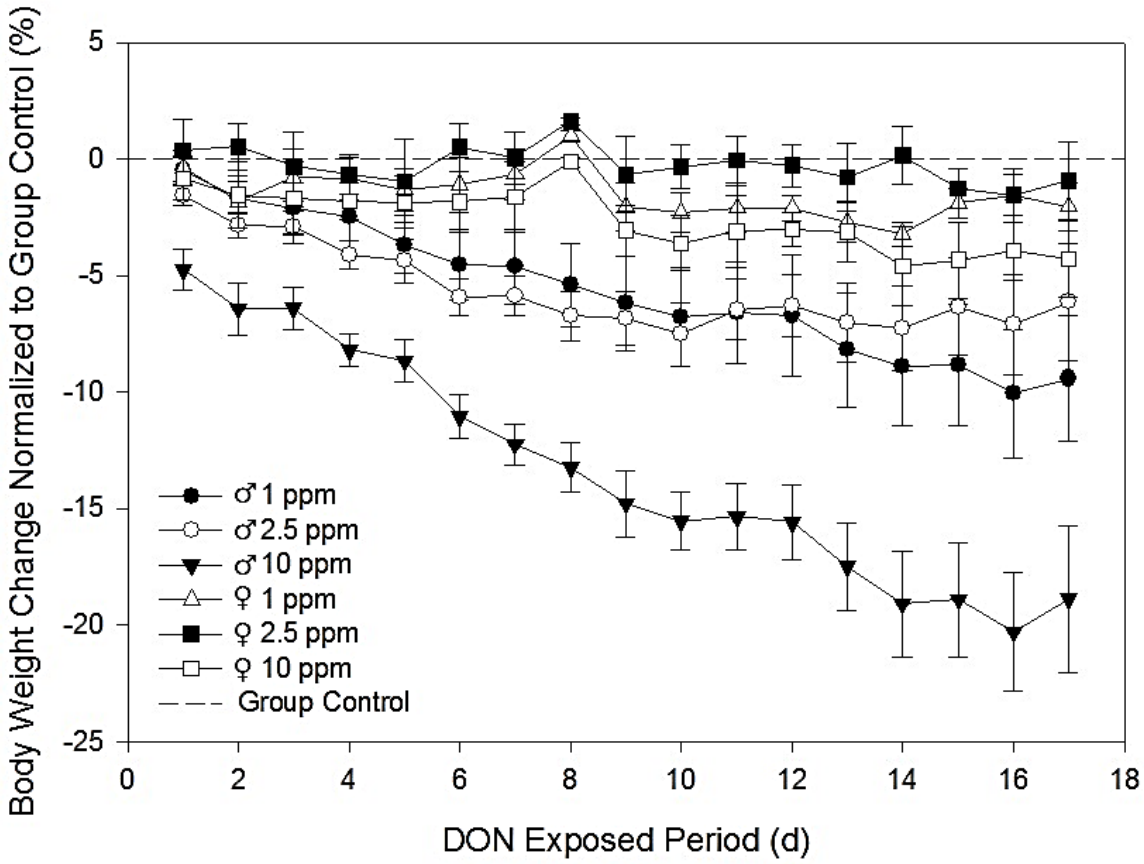


Figure 2.6 Male mice are more sensitive to DON-induced anorexia than female mice upon dietary DON exposure. Changes in daily body weights were determined from weight at study beginning and normalized to group control weight gain. Data are mean $\pm$ SEM (n = 6/group). Statistical significance is indicated in Table 2.3.

Table 2.3 Statistical analysis of Study 3 in Figure 2.6.

| | **Day** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| ♂ **1 ppm** | - | - | - | - | - | - | - | - | a, b |
| ♂ **2.5 ppm** | - | - | - | - | - | - | - | - | a, b |
| ♂ **10 ppm** | - | - | - | a, b | a, b | a, b | a, b | a, b | a, b |
| | | | | | | | | | |
| **Treatment** | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| ♂ **1 ppm** | a | a, b | - | a, b | a | - | - | a, b | a |
| ♂ **2.5 ppm** | a, b | a, b | - | a, b | - | - | - | - | - |
| ♂ **10 ppm** | a, b | a, b | a, b | a, b | a, b | a, b | a, b | a, b | a, b |

Statistically significant differences are indicated as follows: a = different from group control body weight percent change and b = different from female body weight percent change at same dose and day ($p < 0.05$). No statistical significance was observed in females treated with DON in comparison to group control.

the toxin are possible explanations for weight loss leveling near the end of the study.

The results presented here are consistent with previous animal studies examining sex differences with dietary DON exposure (Cote *et al.*, 1985; Greene *et al.*, 1994b; Rotter *et al.*, 1994; Iverson *et al.*, 1995; Andretta *et al.*, 2012). In a study conducted by Cote et al. (1985) in swine, researchers found that castrated male swine (barrows) on diets containing 3.1 ppm and 5.8 ppm DON had increased difficulty with consistent weight gain in comparison with female swine. That study also reported that male swine did not resume normal growth rates after being removed from treatment diets, while female swine growth rates recovered. A meta-analysis found an average weight reduction of 34% in DON treated barrows, while female weights were only reduced by 2% (Andretta *et al.*, 2012). Rotter et al. 1994 found that male ICR mice on diets containing 2-8 ppm DON showed a greater suppression of weight gain than females over a 14 d period compared with control animals. They also reported that animals appeared to show adaptation to the DON diets in the second week of exposure. A 2 year feeding study conducted by Iverson et al. (1995) examined sex differences to prolonged DON exposure in B6C3F1 mice. While they did not observe greater weight suppression in male mice compared with female mice, the study did report that males fed diets containing 5 and 10 ppm DON did consume significantly less than control males and no significant differences in food consumption were reported in female mice.

*2.3.3 Dietary DON exposure contributes to elevated liver DON equivalents in male mice*

DON equivalents in the organs and plasma after 17 d of dietary DON exposure were compared in male and female mice. Liver DON equivalents in male mice fed 1 and 10 ppm DON diets were significantly higher than females by 2.9 and 1.5 fold respectively (Figure 2.7). DON equivalents in the livers of males fed 2.5 ppm DON diets were 1.6 fold higher than the

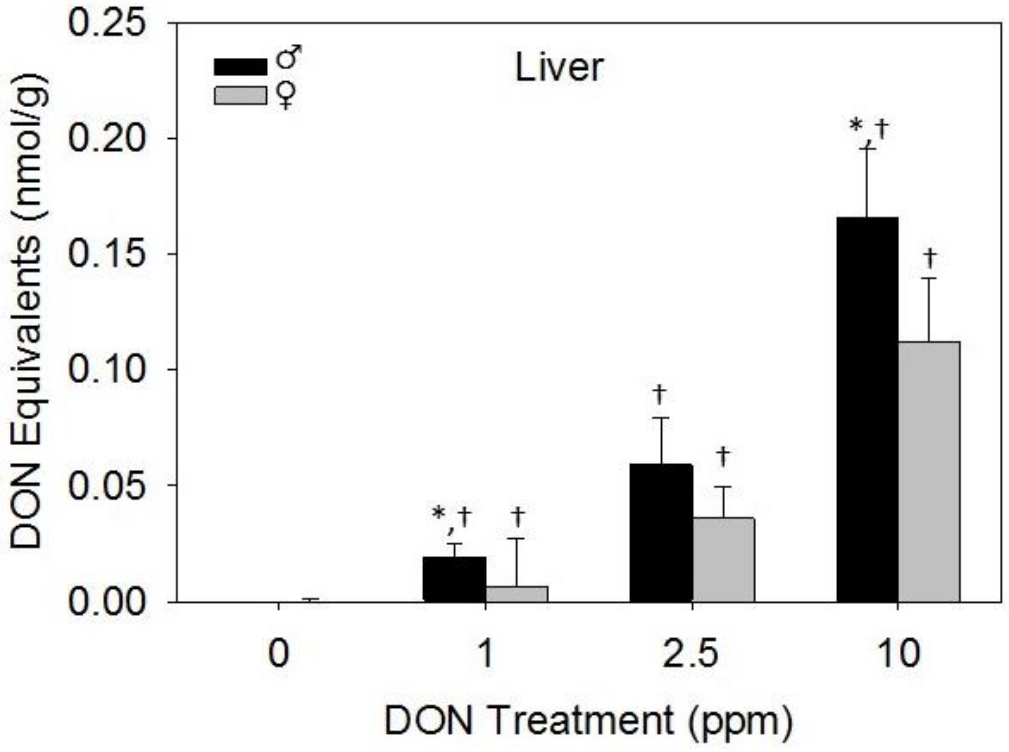


Figure 2.7 Male mice have higher liver DON equivalents than females after dietary toxin exposure. Data are mean ± SEM (n = 6/gp). Asterisk indicates statistical significance from female on same treatment diet and dagger indicates significance from preceding treatment dose within sex ($p < 0.05$).

concentrations found in females, although this difference was not statistically significant. Liver DON equivalents significantly increased with diets containing higher amounts of DON in both male and female mice. Sex differences in toxin concentrations in all other organs analyzed were not statistically significant, with the exception of kidney DON equivalent concentrations being higher in females than males fed 1 ppm DON diets (Figure 2.8). As animals were allowed *ad libitum* access to food prior to euthanasia, the amount of toxin consumed and time of consumption are unknown. While unrestricted access to feed prior to euthanasia complicates data interpretation, identifying higher levels of DON equivalents in male mouse livers in both the acute i.p. exposure Study 1 and Study 3 also could suggest that male mice may absorb more toxin and/or require more time to metabolize the toxin.

*2.3.4 Proinflammatory plasma cytokines were not detectable after 17 d of dietary DON exposure*

Plasma levels of the proinflammatory cytokines IL-6, IL-1β, and TNF-α were not detectable in either male or female mice after 17 d of dietary DON exposure at any treatment level. These findings are consistent with a previous study also reporting inability to detect IL-6, IL-1β, or TNF-α in plasma of mice exposed to 2 mg/mL DON in drinking water for 36 d (Choi *et al.*, 2013).

## 3. Experimental Section

*3.1 Animals*

The Institutional Animal Care and Use Committee at Michigan State University approved all animal experiments. Adult male and adult female C57BL6 mice (12 wk) were purchased from Charles River Laboratories (Portage, MI). Mice were housed singly in polycarbonate cages with

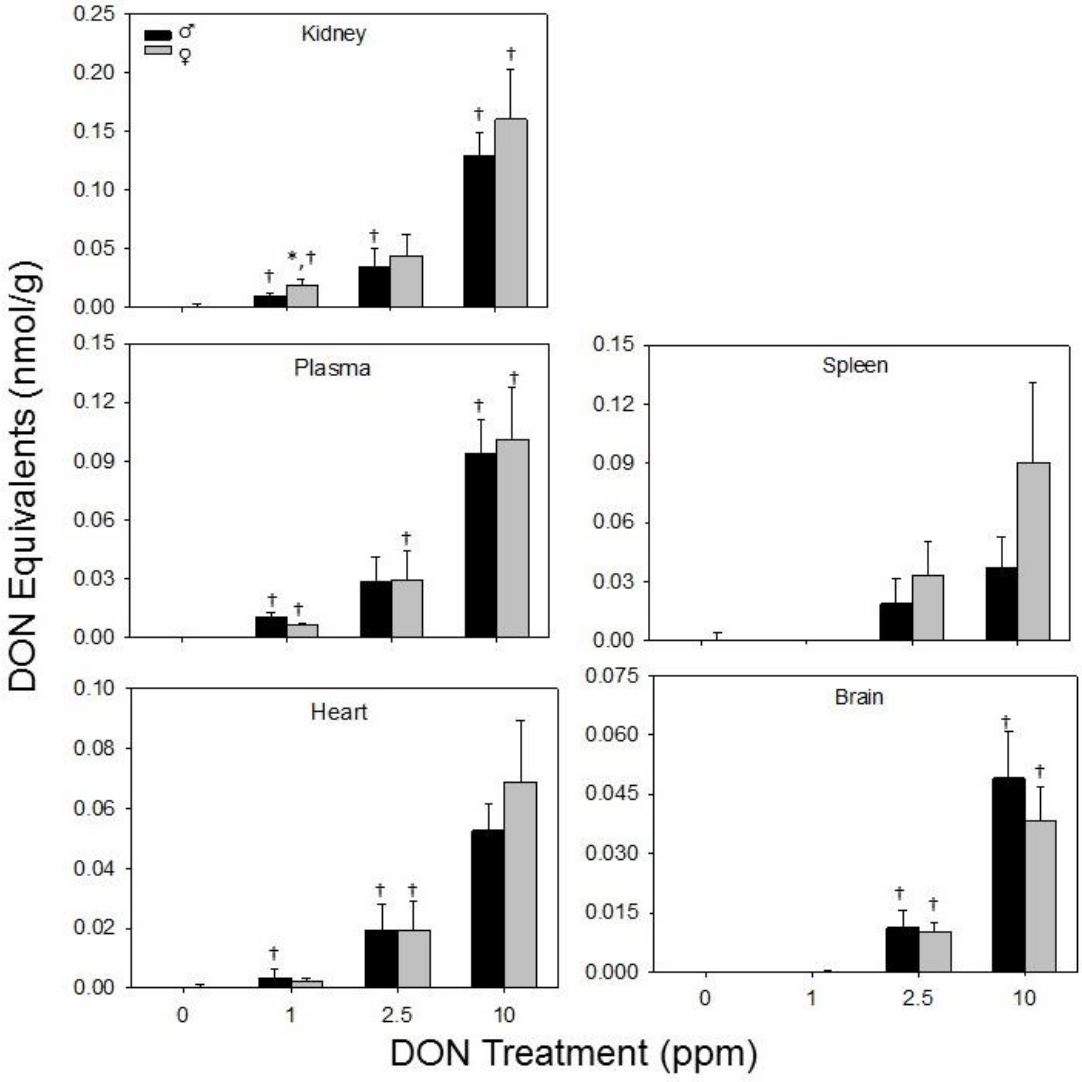


Figure 2.8 Organ DON equivalents increase in dose dependently after dietary DON exposure in mice. Data are mean ± SEM (n = 6/gp). Asterisk indicates statistical significance from male on same treatment diet and dagger indicates significance from preceding treatment dose within sex ($p < 0.05$).

sifted aspen bedding on a 12 hr light/dark cycle, with constant temperature (21-24$^0$C) and humidity (40-55%). In all experiments mice were fed high fat pellet diet (45% kcal from fat; Research Diets, Inc., New Brunswick, NJ) 1 wk prior to DON exposure. High fat diet was used as it was previously determined to provide the most efficient food recovery (Flannery *et al.*, 2011).

*3.2 DON*

DON used for i.p. injections and DON amended diet was obtained from Dr. Tony Durst (University of Ottawa, Canada) and purity was verified to be 98% by elemental analysis (Galbraith Labs, Knoxville, TN). For all i.p. injections, DON was appropriately dissolved in Dulbecco's phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) to yield 100 µL injection volumes. High fat pellet diets containing 0, 1, 2.5 and 10 ppm DON were formulated by Research Diets, Inc. DON concentration of diets were confirmed using the Veratox high sensitivity (HS) enzyme-linked immunosorbent assay according to manufacturer protocol (ELISA; Neogen, Lansing, MI).

*3.3 Experimental design*

*3.3.1 Study 1*

The effects of sex on food intake after acute i.p. DON exposure were assessed as illustrated in Figure 1. Upon arrival, mice were acclimated to food and handling according to our previously described feed refusal assay (Flannery *et al.*, 2011). After acclimation, male and female mice (n= 10/group) were fasted from 10:00 to 18:00 h, and i.p. injected with either 0 (PBS vehicle control), 1 or 5 mg/kg bw DON (Figure 2.1). Food consumption was measured hourly 1 to 7 h post exposure, and at 12, 20, 24 and 36 h post exposure. Measurements were conducted under red light conditions during dark cycle.

*3.3.2 Study 2*

The effects of sex on tissue DON equivalent concentrations and plasma concentrations of proinflammatory cytokines and satiety hormones were measured after acute i.p exposure to the toxin. Male and female mice (n = 5-6/group), were similarly fasted as in Study 1 and exposed to 1 mg/kg bw DON in PBS or PBS vehicle via i.p. injection. Mice were euthanized via $CO_2$ chamber at 1, 2, and 4 h post exposure without food replacement. Blood was collected via cardiac puncture and the kidney, liver, spleen, heart and brain were collected and immediately snap frozen. Plasma was isolated from blood by centrifugation at 3,500 x g for 10 minutes at 4°C. Plasma and organs were stored at -80°C until analysis.

*3.3.3 Study 3*

The effects of sex on response to dietary DON exposure were assessed. After acclimation, male and female were randomized into equal weight groups by sex (n= 6/group) and placed on high fat diets containing 0, 1, 2.5 and 10 ppm DON. Body weights were measured daily at 10:00 am for 17 d. The study was terminated at 17 d as body weight changes appeared to be stabilizing at this point. Food intake measurements were attempted the entire 17 d period. However after 2 d, mice on diets containing DON progressively began to shred the pellets into fine particles that were unrecoverable from sieved bedding and precluded accurate food recovery. After 17 d of DON exposure, mice were euthanized via $CO_2$ chamber at 8:00 am. Food access to treatment diets was *ad libitum* prior to euthanasia. Blood was collected via cardiac puncture and the kidney, liver, spleen, heart and brain were collected and immediately snap frozen. Plasma was isolated from blood. Plasma and organs were stored at -80°C until analysis.

*3.4 Analytical*

*3.4.1 DON quantification*

DON quantification in plasma and organs were analyzed using the Veratox high sensitivity (HS) ELISA (Neogen, Lansing, MI) as described previously with slight modifications (Pestka *et al.*, 2008). Briefly, organs were homogenized 1:1 in PBS (except the heart, which was homogenized 1:2). Tissue homogenates were heated at 100°C for 5 min and then centrifuged at 14,000 g for 10 min at 4°C. The resulting supernatant was analyzed for DON using a F3 ELISA plate reader at 650 nm and Softmax software (Molecular Devices, Menlo Park, CA). DON is reported as DON equivalents as the ELISA was found to be completely cross reactive with DON3GlcA obtained from Dr. Philipp Fruhmann (Vienna University of Technology, Austria) (Figure 2.3).

*3.4.2 Proinflammatory cytokine analyses*

Plasma levels of the proinflammatory cytokines IL-6, IL-1β, and TNF-α were determined using Duoset ELISAs from R&D Systems (Minneapolis, MN). Equal volumes of mouse plasma samples (n = 5-6/group Study 2; n = 6/group Study 3) were pooled within groups and ran in technical replicates (n = 4 rep) because volumes of individual samples were low.

*3.4.3 Satiety hormone analyses*

Plasma levels of the gut satiety hormones CCK and PYY3–36 in with plasma samples from Study 2 (n = 6/group) were analyzed using ELISA kits for CCK (CCK [26–33], nonsulfated; human-, rat-, and mouse-specific) and PYY (PYY [3–36]; mouse-, rat-, porcine-, and canine-specific) (Phoenix Pharmaceuticals, Burlingame, CA). CCK and PYY were not measured in Study 3 as animals had *ad libitum* access to food prior to study termination.

*3.5 Statistical analysis*

Statistical analysis was conducted by SigmaPlot version 11.0 (Jandel Scientific; San Rafael, CA). Statistical comparisons between sex made at each time point using a Student's t-test, unless normality failed. If normality was not met, a Mann-Whitney Rank Sum test was performed. Statistical comparisons between sex and dose were made at each time point using a one-way analysis of variance (ANOVA), unless normality failed. A Kruskal–Wallis one-way analysis of variance by ranks was performed if normality was not met. Student–Neuman–Keuls was used in all post-hoc analysis for parametric and non-parametric animal groups of equal numbers. Dunn's post-hoc analysis was used in non-parametric analysis of animal groups with unequal numbers. Pearson product movement correlations were performed to determine statistical significance between food consumption and treatment levels of DON diets by sex and day in Study 3. Differences were considered significant when $p < 0.05$.

## 4. Conclusions

Our data indicate that male mice were more sensitive than females to anorexia induction after acute and dietary DON exposure. These effects corresponded to decreasing body weight in males fed DON-containing diets. As toxin organ clearance was slower in male mice than female mice, future studies should evaluate possible sex differences in DON uptake, metabolism and excretion. Relating sex differences to increased vulnerability to DON will be important to accurate risk assessment of this mycotoxin or other trichothecenes.

# CHAPTER 3: High Sensitivity of Aged Mice to Deoxynivalenol (Vomitoxin)-Induced Anorexia Corresponds to Elevated Proinflammatory Cytokine and Satiety Hormone Responses

Data in this chapter has been published in Clark, E.S., Flannery, B.M., Gardner, E.M., and Pestka, J.J. (2015). High sensitivity of aged mice to deoxynivalenol (vomitoxin)-induced anorexia corresponds to elevated proinflammatory cytokine and satiety hormone responses. *Toxins*, 7(10), 4199-4215.

## Abstract

Deoxynivalenol (DON), a trichothecene mycotoxin that commonly contaminates cereal grains, is a public health concern because of its adverse effects on the gastrointestinal and immune systems. The objective of this study was to compare effects of DON on anorectic responses in aged (22 mos) and adult (3 mos) mice. Aged mice showed increased feed refusal with both acute i.p. (1 mg/kg bw and 5 mg/kg bw) and dietary (1, 2.5, 10 ppm) DON exposure in comparison to adult mice. In addition to greater suppression of food intake from dietary DON exposure, aged mice also exhibited greater body weight suppression. When aged mice were acutely exposed to 1 mg/kg bw DON i.p., aged mice displayed elevated DON tissue levels and delayed clearance in comparison with adult mice. Acute DON exposure also elicited higher proinflammatory cytokine and satiety hormone responses in the plasma of the aged group compared with the adult group. Increased susceptibility to DON-induced anorexia in aged mice relative to adult mice suggests that advanced life stage should be considered when conducting risk assessments on DON and other trichothecenes.

## 1. Introduction

Deoxynivalenol (DON, vomitoxin), a trichothecene mycotoxin produced by the fungus *Fusarium graminearum*, is a common cereal grain contaminant that is highly resistant to heat processing, leading to contamination of human and animal food (Pestka, 2010b). Adverse effects of acute exposure to DON include anorexia, diarrhea, and vomiting in experimental animals (Pestka, 2010a). In addition, chronic DON exposure can lead to immunotoxic effects and growth retardation. Mice, commonly used in experimental studies for DON risk assessment, are incapable of vomiting but exhibit feed refusal and body weight suppression following exposure to the toxin (Pestka and Amuzie, 2008; Andretta *et al.*, 2012).

Previous studies have investigated sex differences in the susceptibility of young animals to DON-induced anorexia (Cote *et al.*, 1985; Rotter *et al.*, 1994; Pestka and Amuzie, 2008; Andretta *et al.*, 2011; Andretta *et al.*, 2012); however, the effects of this toxin on aged animals is largely unaddressed. Studying the adverse effects of DON in advanced life stage animals is important because approximately 25% and 30% elderly men and women, respectively, are reported to be in an anorectic state (Thomas, 2009; Donini *et al.*, 2011; Martone *et al.*, 2013). This phenomenon of unintentional weight loss in advanced life stage has been termed the "anorexia of aging" and is a high predictor of morbidity and mortality in the elderly (Morley and Silver, 1988; Landi *et al.*, 2010). The etiology of anorexia is complex and thought to be caused by many factors including depression, increased susceptibility to illness, diet, and the burden of multiple medications (Chapman *et al.*, 2002; Mangoni and Jackson, 2004; Thomas, 2009). In a study of elderly Finnish men with depressive symptoms (65-84 years of age; n = 688), many reported gastrointestinal complaints over a 2 wk period including diarrhea (20%), stomach pains (37%), nausea (29%), vomiting (9%), and loss of appetite (21%) (Kivela *et al.*, 1988). As these

symptoms are also present with DON intoxication, it is possible that elderly individuals who are already predisposed to gastrointestinal aliment might be more sensitive to the negative effects of DON.

One potential cause of DON-induced anorexia is the induction of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, which have been previously shown to cause sickness behavior in humans and experimental animals (Dantzer, 2001; Girardet *et al.*, 2011; Lebrun *et al.*, 2015). Our lab has reported the induction of these cytokines in female mice, as well as an increased plasma IL-6 response in male mice in comparison with female mice (Azcona-Olivera *et al.*, 1995; Pestka and Amuzie, 2008; Clark *et al.*, 2015b). Prior investigations of the effect of lipopolysaccharide (LPS) in aged and adult mice have shown that the former exhibit a greater cytokine response to LPS than that of adults (Saito *et al.*, 2003; Huang *et al.*, 2008). Huang *et al.* (2008) found that plasma IL-6 levels of aged mice were 2- and 6.6-fold higher at 2 and 8 h post intracerebroventricular (i.c.v.) injection of 10 ng of LPS than adult mice (Huang *et al.*, 2008). Another study found that plasma levels of IL-1β and IL-6 were significantly increased in aged mice in comparison with adults at 6 h post exposure to 5 μg/g bw LPS administered by intraperitoneal (i.p.) injection (Saito *et al.*, 2003).

Additionally, our lab discovered that the gut satiety hormones peptide YY (PYY) and cholecystokinin (CCK) are induced in mice upon i.p. and oral DON exposure and both contribute to feed refusal caused by this mycotoxin (Flannery *et al.*, 2012; Wu *et al.*, 2014b). When Stanström *et al.* (1998) compared the number of enteroendocrine cells responsible for PYY secretion in the intestinal epithelium of mice that were 3 and 24 months of age, they found that aged mice had significantly more of these cells (Sandstrom *et al.*, 1998). Another study in rats revealed that PYY-containing cells per colonic crypt increased with age (Sweet *et al.*, 1996). In

a study comparing the effects of CCK in adult and aged mice, researchers found that aged mice showed increased sensitivity to CCK and that the action of CCK was prolonged in aged mice (Silver *et al.*, 1987). As PYY and CCK are induced by DON and tend to increase with age, exploring the combined effects of DON exposure and aging is of importance.

The aims of this study were to compare feed refusal responses between adult and aged mice after acute and dietary DON exposure. Study 1 compared feed refusal differences by age following acute i.p. DON exposure. In Study 2, we compared DON tissue concentrations, proinflammatory plasma cytokine responses, and satiety hormone responses in adult and aged animals following acute i.p. exposure to the toxin. Finally, Study 3 compared differences in food intake and body weight suppression upon dietary DON exposure in adult and aged mice. The results presented herein indicate that in aged mice the food refusal response is greater than adult mice following both acute i.p. and dietary DON exposure and that acute exposure is accompanied by prolonged DON tissue clearance as well as increased proinflammatory cytokine and gut satiety hormone responses in aged mice.

## 2. Results

### *2.1 Study 1*

#### *2.1.1 DON-induced feed refusal is greater in aged mice than adult mice*

The effects of acute i.p. exposure to 1 mg/kg and 5 mg/kg bw DON were compared in aged and adult mice over 36 h (Figure 3.1). Aged mice treated with 1 mg/kg DON consumed less food than control aged mice from 1 h to 36 h post-injection (PI) (Figure 3.2). In

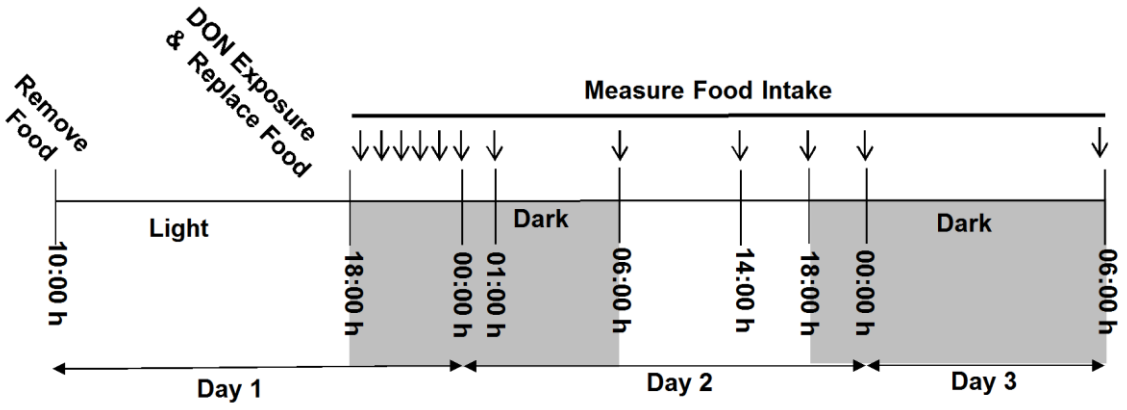


Figure 3.1 Study 1 experimental design. On day 1, mice were fasted from 10:00 to 18:00 h then exposed to DON treatments or vehicle control. Food was immediately replaced following exposure and food measurements were recorded hourly from 1 to 7 h PI, and at 12, 20, 24, and 36 h PI as indicated by arrows.

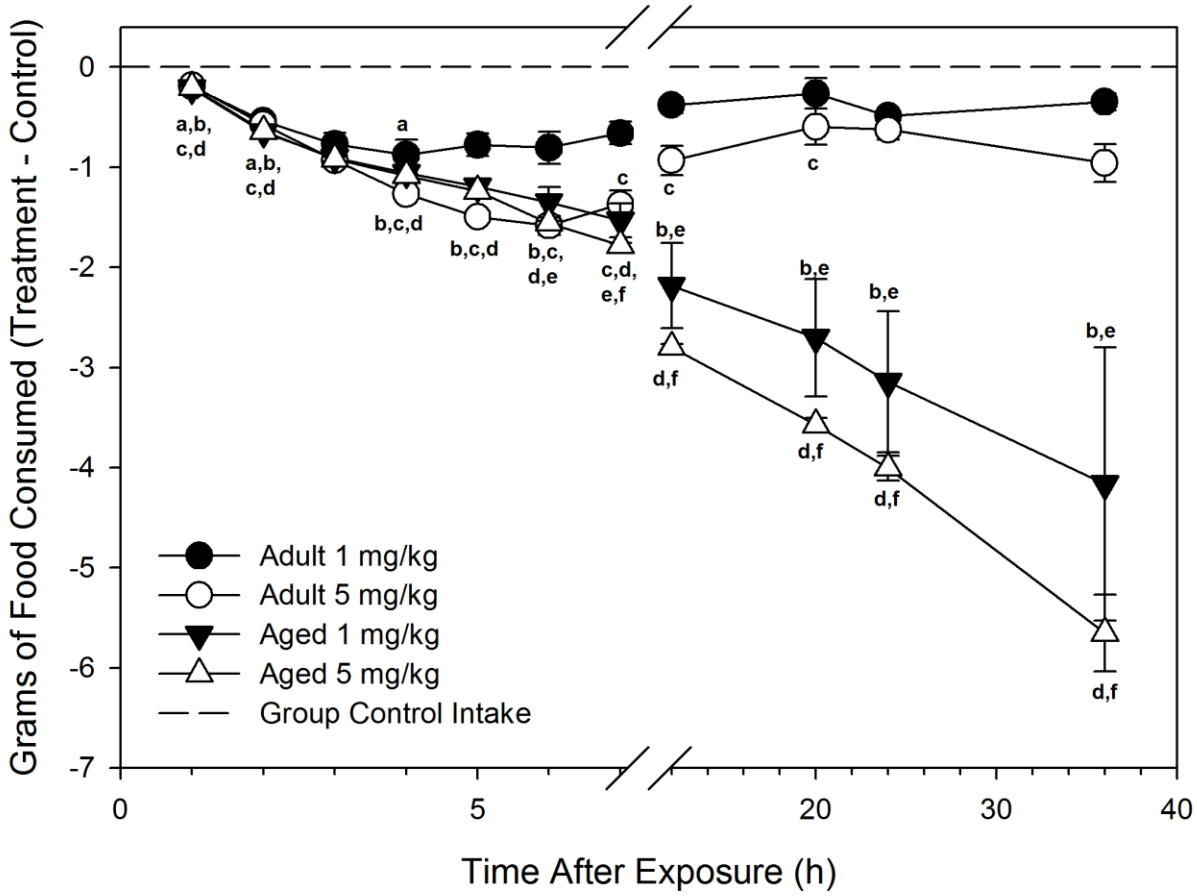


Figure 3.2 Aged mice are more sensitive to DON-induced feed refusal than adults following acute i.p. exposure. Food intake is cumulative at time point and was normalized to group control average. Data are mean $\pm$ SEM (n = 5/gp). Significant differences ($p < 0.05$) are indicated as follows: a = 1 mg/kg bw DON dosed adult different from adult control; b = 1 mg/kg bw DON dosed aged different from aged control; c= 5 mg/kg bw DON dosed adult different from adult control; d = 5 mg/kg bw DON dosed aged different from aged control; e = aged different from adult at 1 mg/kg bw DON; and f = aged different from adult at 5 mg/kg bw DON.

comparison, adult mice treated with the same dose of DON consumed less food than adult controls at 1 to 4 h PI and then began to show recovery in food intake. By 6 h PI, aged mice exposed to 1 mg/kg DON ingested significantly less food than adult mice. At this dose, aged mice consumed 82% less food than aged control mice, while adult mice at this dose consumed 41% less food than adult controls at this time point.

Following exposure to 5 mg/kg bw DON, total food consumption was reduced from 1 to 20 h PI and 1 to 36 h PI for adult and aged mice, respectively. At 7 h PI, 5 mg/kg bw DON-treated adult mice consumed 32% feed of adult vehicle controls, whereas DON-treated aged mice ate only 2% of those of aged vehicle controls.

At 36 h PI, aged mice continued to exhibit a substantial depression in food intake compared to aged control mice with food consumption accounting for 43% and 22% of that of control aged mice at 1 mg/kg and 5 mg/kg bw DON, respectively. In comparison, food intake in adult mice treated with 1 mg/kg and 5 mg/kg bw DON at 36 h PI was 94% and 84% respectively of adult control food intake suggesting that this group has nearly fully recovered from the toxin.

*2.2 Study 2*

*2.2.1 DON tissue concentrations are higher in aged mice compared with adults after acute exposure*

When DON tissue concentrations in kidney, liver, plasma, spleen, heart and brain were assessed over 12 h following i.p. exposure to DON at 1 mg/kg bw, toxin levels were consistently higher in aged mice than in adult mice (Figure 3.3). The initial rank order of DON concentrations in both aged and adult mice was: kidney>liver>plasma>spleen>heart>brain. At 1 h PI, aged mice had higher DON concentrations in the kidney (1.8-fold increase) and plasma

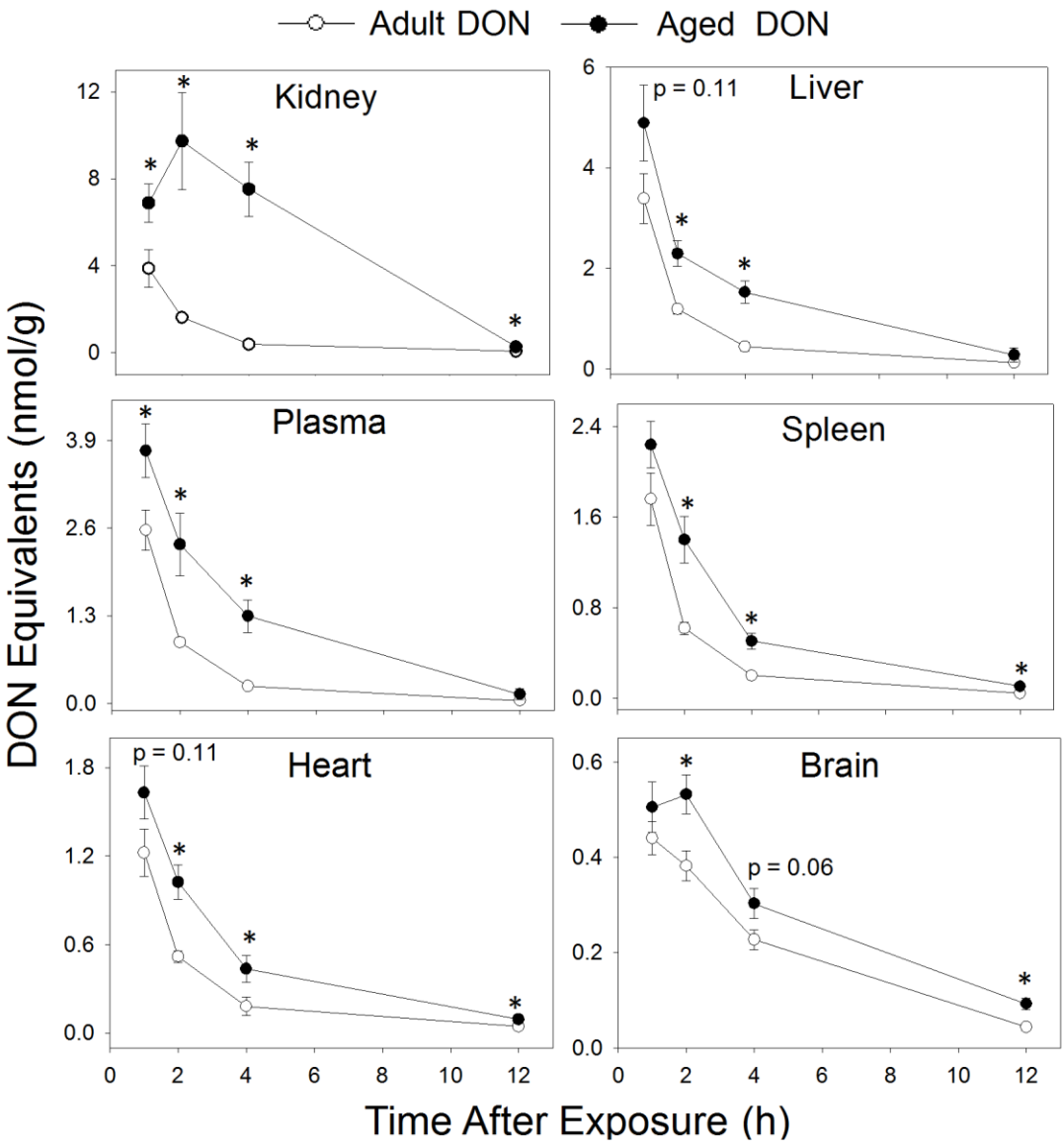


Figure 3.3 DON equivalent concentrations are higher in aged mice than adult mice post-acute i.p. exposure to 1 mg/kg bw DON. DON is reported as DON equivalents because the Veratox hs ELISA was fully cross reactive with DON3GlcA. Control animals did not have detectable concentrations of DON (data not shown). Data are mean ± SEM (n = 8-10/gp). Asterisk indicates statistical significance from adult at time point ($p < 0.05$).

(1.5-fold increase). At 2 h PI, DON concentrations were higher in all tissues of aged mice compared with adults, ranging from 6-fold higher in the kidney to 1.5-fold higher in the brain. Interestingly, DON concentrations in kidneys and brains of aged animals were higher at 2 h PI than at 1 h PI. At 4 h PI, DON concentrations were significantly higher in all organs of aged mice except the brain, with the largest differences existing in the kidney (20-fold higher) and plasma (5-fold higher). Aged mice continued to exhibit higher DON levels in the kidney, spleen, heart, and brain at 12 h PI, with concentrations ranging from 4.3 to 2-fold above the levels of adult mice.

*2.2.2 Plasma IL-6 and IL-1β are higher in aged mice compared with adults after acute DON exposure*

When the effects of acute exposure to 1 mg/kg bw DON on plasma proinflammatory cytokines were assessed, aged mice exhibited higher plasma levels of IL-6 and IL-1β than adult animals (Figure 3.4) . Beginning at 2 h PI, aged mice treated with DON had a 2.2-fold increase in both plasma IL-6 and IL-1β when compared with adult mice at 2 h PI. At 4 h PI, aged mice had a 120-fold difference of IL-6 in comparison with adult animals, which had nearly returned to adult control levels at this time point. Aged animals also had a 4.4 fold increase in IL-1β at 4 h PI when compared with adult mice. Plasma IL-6 levels remained significantly elevated in aged mice when compared with control aged mice and DON-treated adult mice at 12 h PI, while IL-1β levels were not statistically significant in any group at this time point. Vehicle treated aged mice had higher plasma IL-6 at 2 and 4 h post exposure than treated adults. However, IL-6 levels were very low compared to DON treated mice at these time points. Adult mice had higher levels of IL-1β at 2 and 4 h PI. TNF-α was not detectable in the plasma of either aged or adult mice at any time point. This latter result is in agreement with a previous study in our lab comparing

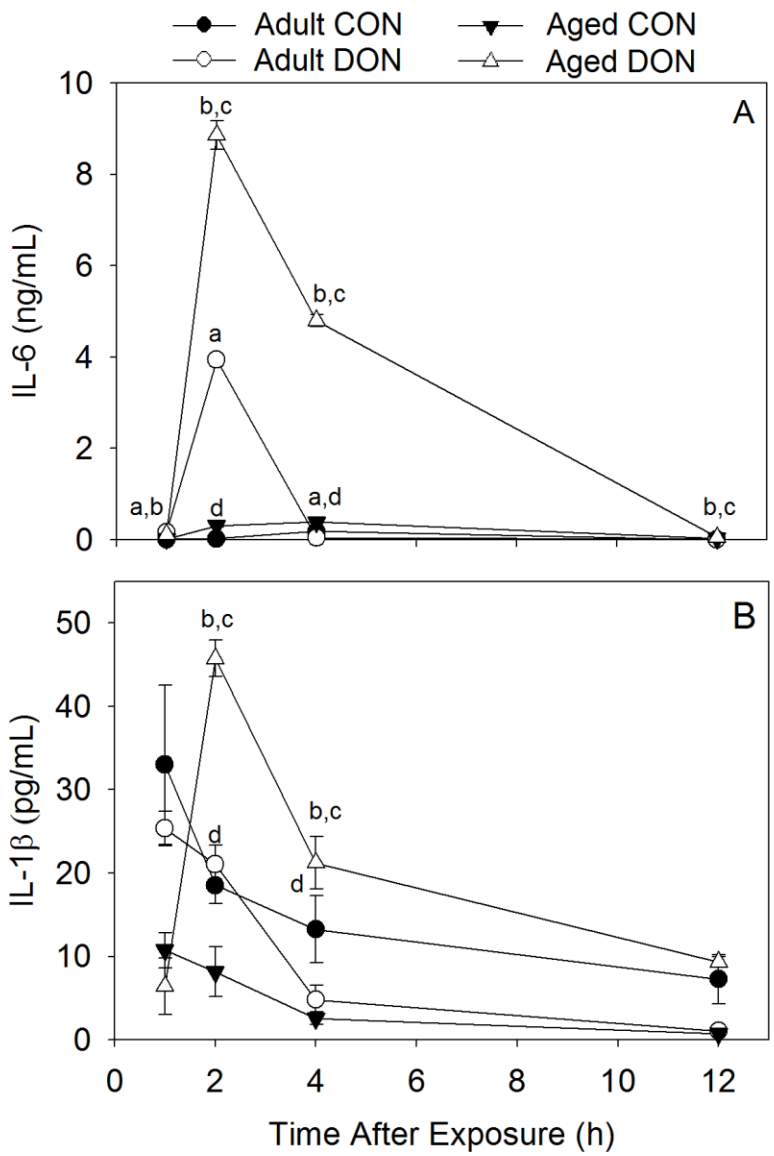


Figure 3.4 Aged mice exhibit higher IL-6 and IL-1β plasma levels post-acute i.p. exposure to 1 mg/kg bw DON. A) IL-6 plasma levels ng/mL and B) IL-1β plasma levels pg/mL. Data are mean ± SEM (n = 4/replicates). Significant differences are indicated as follows: a = DON dosed adults different from adult control; b = DON dosed aged different from aged control; and c = DON dosed aged different from DON dosed adult ($p < 0.05$); d = vehicle control age groups different.

proinflammatory cytokines in male and female mice treated with 1 mg/kg bw DON (Clark *et al.*, 2015b).

### *2.2.3 DON induction of PYY and CCK is greater in aged animals than adults with DON exposure*

When the effects of acute exposure to 1 mg/kg bw DON on plasma levels of gut satiety hormones were measured, DON elicited greater increases in PYY and CCK in aged mice than adult mice (Figure 4.5). DON-treated aged mice exhibited a trend toward greater PYY than similarly treated adult mice at 1 and 2 h PI. At 4 h PI, aged mice treated with DON exhibited a robust increase in plasma PYY, having levels that were 51% higher than control aged mice. In contrast, adult mice treated with DON at this time point were not different from control adults. Plasma PYY had returned to baseline levels by 12 h PI in both DON-treated groups. In mice exposed to vehicle only, PYY plasma levels trended higher in aged mice than adults and PYY was significantly higher in control aged mice at 2 and 4 h PI than control adults.

DON-treated aged mice exhibited a trend toward greater CCK than similarly treated adult mice at 1 and 4 h PI. At 1 h PI, aged mice exposed to DON had a 99% increase in plasma CCK in comparison to control aged animals and were significantly higher than control aged mice and DON-treated adults. In comparison, while adult mice exhibited a 59% increase in CCK over adult controls at 1 h PI, they were not significant higher than control adults. DON-treated animals continued to show trends toward increased plasma levels of CCK at 2 and 4 h PI, although these were not significant. At 12 h PI, CCK concentrations were no longer impacted by DON in either age group. CCK plasma levels were higher in vehicle treated aged mice than adults, though not significantly.

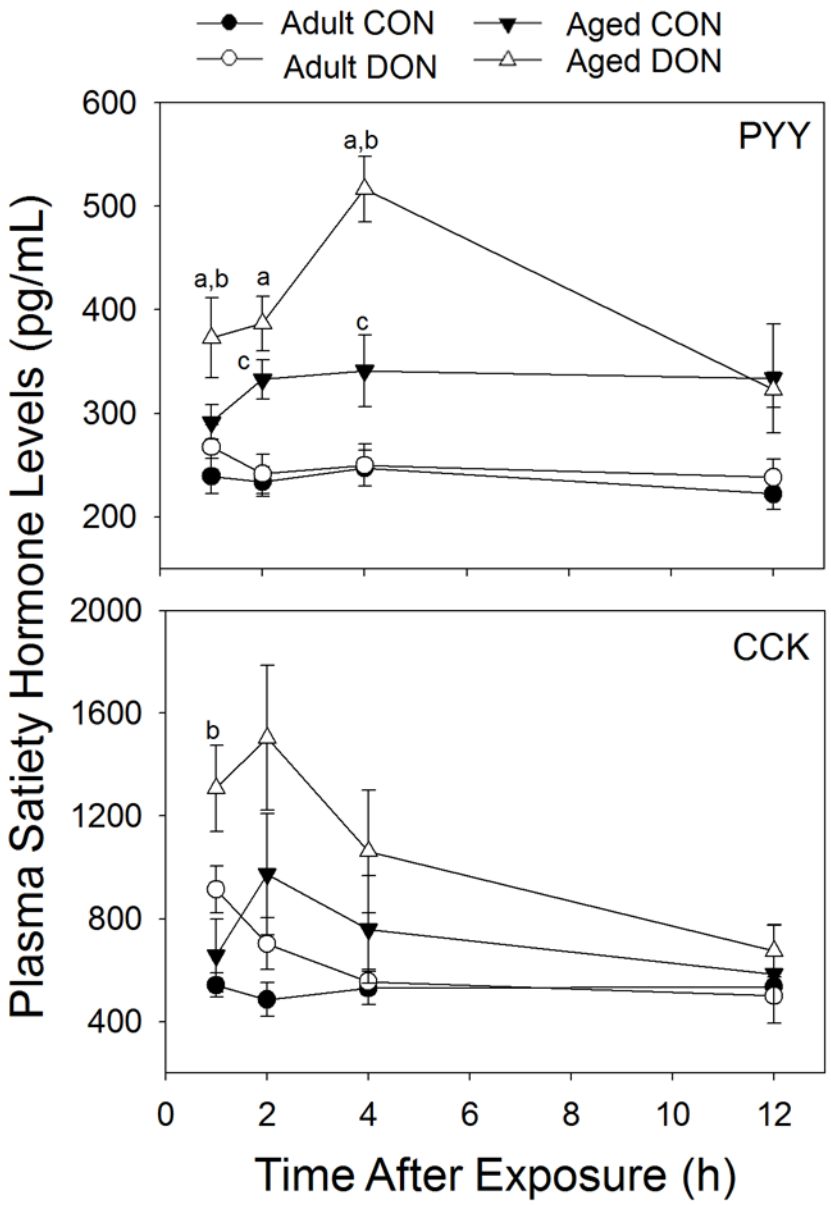


Figure 3.5 DON induction of plasma PYY and CKK is greater in aged mice after acute i.p. exposure. Data are mean ± SEM (n = 8-10/gp). Significant differences ($p < 0.05$) are indicated as follows: a = DON dosed aged different from DON dosed adult; b = DON dosed aged different from aged control; and c = control aged different from control adult.

*2.3 Study 3*

*2.3.1 Reduction in food intake is greater in aged mice than adults with dietary DON exposure*

The impact of dietary exposure to DON at 0, 1, 2.5, and 10 ppm on food intake were compared between aged and adult mice. Aged mice showed a greater reduction in food intake upon dietary DON exposure than adult mice (Table 3.1). After 1 d of dietary DON exposure, both aged and adult animals exhibited a statistically significant negative correlation between decreasing food intake with increasing DON concentration in the diet. Aged mice fed DON diets containing 2.5 and 10 ppm consumed 9% and 62% less food respectively than aged control mice over the first day, while adult mice fed the same concentration diets consumed 2% and 58% less food than adult controls, respectively, at this time point. After 2 d, aged mice continued to exhibit decreasing food intake with increasing DON concentration while adult mice no longer exhibited this negative correlation. Aged mice fed diets containing 2.5 and 10 ppm DON consumed 13% and 54% less food respectively, than aged control mice at d 2, while adult mice fed the same concentration diets consumed 3% and 10% less food than adult controls at this time point.

*2.3.2 Dietary DON causes greater suppression of weight gain in aged mice*

Effects of consuming feed containing 1, 2.5, and 10 ppm DON on body weight suppression were further compared by age. Aged mice were more sensitive to body weight suppression than adults (Figure 3.6). Aged mice fed diet containing 2.5 ppm DON weighed less than group control mice and adult mice fed the same diet beginning on d 6 of the exposure period, weighing 5% less than aged control mice while adult treated mice weighed 0.4% less than adult control mice. At the study termination (d 14), aged mice fed 2.5 ppm DON weighed

Table 3.1 Reduction in food consumption is greater in aged mice fed DON containing diet.

| Age Group | Conc. DON Diet ppm | % Control Food Intake 0-24 h | Correlation Coefficient | % Control Food Intake 24-48 h | Correlation Coefficient |
|---|---|---|---|---|---|
| **Adult** | 1 | 103.8 ± 9.1 | | 97.8 ± 7.3 | |
| **Adult** | 2.5 | 98.3 ± 8.1 | r = -0.848 | 96.7 ± 5.1 | r = -0.275 |
| **Adult** | 10 | 42.4 ± 6.9 | p = 0.000002 | 90.4 ± 7.3 | p = 0.274 |
| **Aged** | 1 | 101.0 ± 5.9 | | 92.7 ± 7.3 | |
| **Aged** | 2.5 | 90.5 ± 13.5 | r = -0.810 | 87.2 ± 8.1 | r = -0.775 |
| **Aged** | 10 | 37.8 ± 5.2 | p = 0.00003 | 45.9 ± 10.6 | p = 0.0001 |

Values are percent of group control food intake. Data are mean ± SEM (n = 4-5/gp).

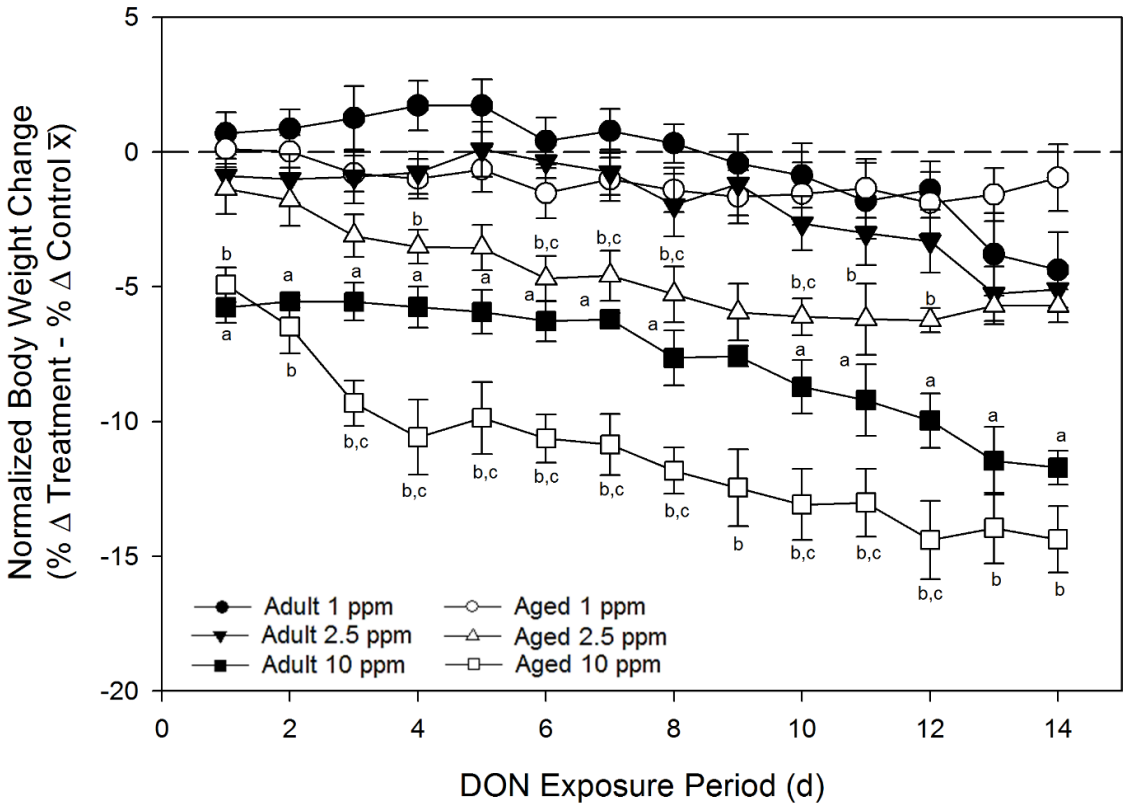


Figure 3.6 Aged mice are more sensitive to DON-induced anorexia than adult mice upon dietary DON exposure. Changes in daily body weights were determined from weight at study initiation and normalized to group control weight gain. Dashed line indicates group control body weight. Data are mean ± SEM (n = 8-10/gp). Statistical significance differences are indicated as follows: a = DON treated adult different from control adult; b = DON treated aged different from control aged; and c = DON treated aged different from DON treated adult fed same ppm DON diet.

6% less than aged control mice. Adult mice fed the same diet weighed 5% less than adult controls at d 14 and significance between differences in weight loss by age no longer existed. Aged mice fed diet containing 10 ppm initially exhibited a rapid depression of weight gain in comparison with controls and on d 4 weighed 11% less than aged control mice. In comparison, adult mice fed 10 ppm DON diet weighed 6% less than adult controls at d 4. After 14 d of exposure to diet containing 10 ppm DON, aged and adult mice continued to show significant weight suppression in comparison to respective group controls, however, significant differences between adult and aged mice were no longer evident upon termination of the study.

*2.3.4 DON tissue concentrations are elevated dose dependently with dietary exposure*

DON tissue concentrations were compared between aged and adult mice after 14 d of dietary DON exposure. Tissue concentrations of DON elevated with increasing DON exposure; however, the increase was only significant in mice fed diets containing 10 ppm DON, with the exception of the DON levels being significantly higher in the spleens of mice fed diet containing 2.5 ppm DON (Figure 3.7). No significant differences in tissue concentrations of DON were observed between aged and adult mice; however, adult mice fed diets containing 10 ppm DON had higher DON concentrations in the spleen than aged animals fed the same diet. As animals were allowed *ad libitum* access to food prior to euthanasia, the amount of toxin consumed and time of consumption are unknown. The observation that aged mice tended to have lower DON levels than adult mice fed the same diet could indicate that aged animals were consuming less food, and thus less toxin, than adults.

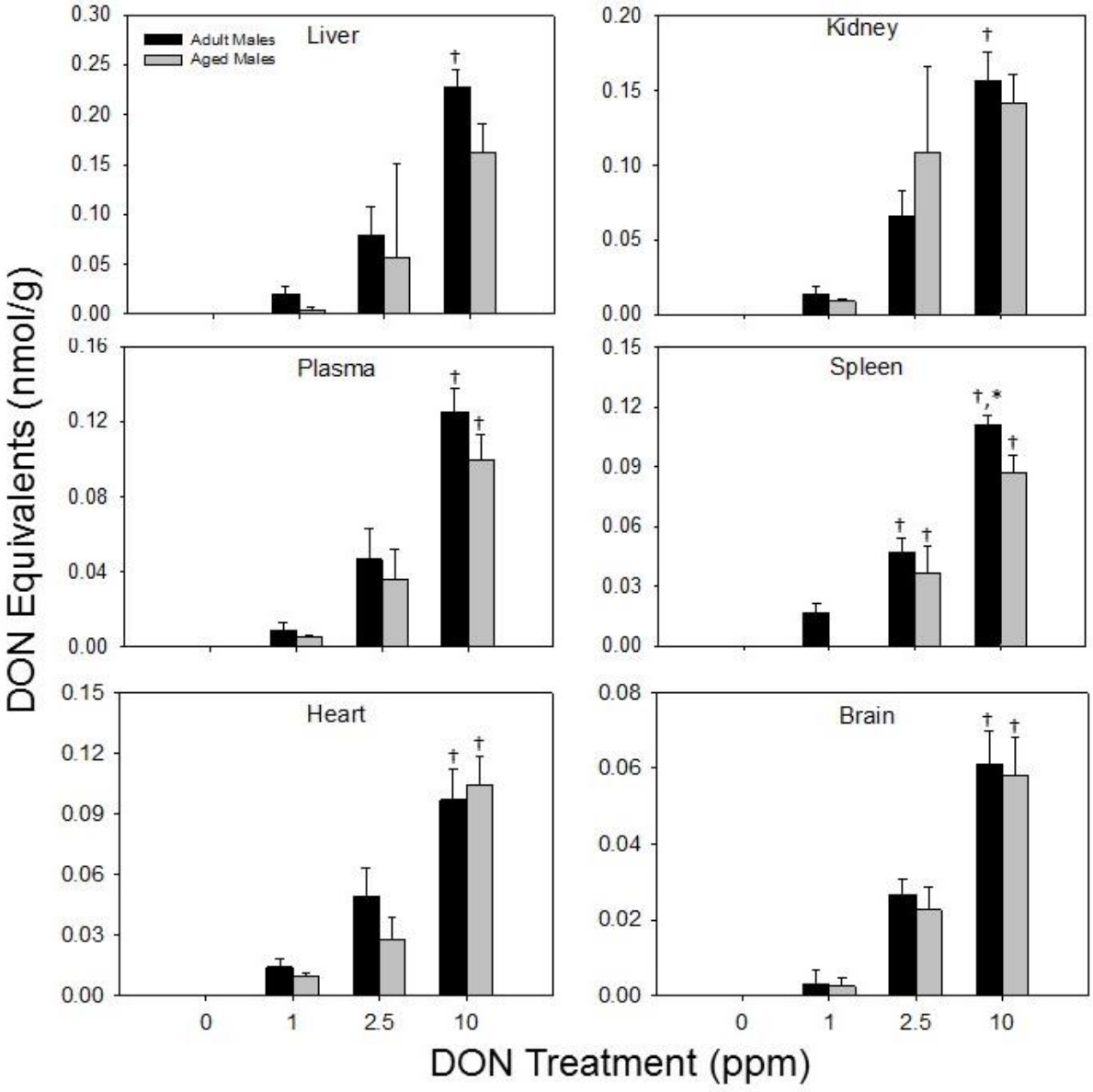


Figure 3.7 Tissue DON concentrations increase in a dose dependent after dietary DON exposure in mice. Data are mean ± SEM (n = 6/gp). Asterisk indicates statistical significance from aged mice on same treatment diet and dagger indicates significance from control diet within age group ($p < 0.05$).

*2.3.5 Proinflammatory plasma cytokines are not detectable after 14 d of dietary DON exposure*

Plasma levels of the proinflammatory cytokines IL-6, IL-1β, and TNF-α were not detectable in aged or adult mice after 14 d of dietary DON exposure at any treatment level. These findings are consistent with previous research in our lab also reporting no change in plasma levels of IL-6, IL-1β, or TNF-α after 17 d of exposure to diets containing 1, 2.5, and 10 ppm DON in either male or female mice (Clark *et al.*, 2015b). A study examining mice exposed to 2 mg/mL DON in drinking water for 36 d could not detect these proinflammatory cytokines in plasma (Choi *et al.*, 2013).

## 3. Discussion

Prior investigations have addressed increased susceptibility of young mice, poultry, and swine to DON, but negative effects experienced by older animals have been largely unstudied (Pestka and Amuzie, 2008; Andretta *et al.*, 2011; Andretta *et al.*, 2012). This study is the first to report increased anorectic responses in aged mice compared with young adults following DON exposure. The discovery that anorectic responses to DON are greater in old animals is critical because advanced age in humans leads to a phenomenon known as the "anorexia of aging" in which elderly individuals show unintended weight loss (Morley and Silver, 1988; Landi *et al.*, 2010; Martone *et al.*, 2013).

The finding that aged mice have higher tissue concentrations of DON following acute exposure over a prolonged period compared with adults suggests that aged mice might differ in their ability to absorb, metabolize, or excrete the toxin. The contention that aged animals have lower capacity to metabolize and thus excrete DON is supported by the previous observation of

lower mRNA levels of UDP-glucuronosyltransferases, the family of enzymes responsible for DON glucuronidation, in aged mice when compared with their adult counterparts (Zhang *et al.*, 2010).

Increased levels of DON in the kidneys of aged mice relative to adults is consistent with the notion that aged animals have a reduced capacity to excrete the toxin. As kidney DON concentrations in aged mice were higher at 2 h PI than at 1 h PI, it appears that the toxin accumulated in the kidney. This result could be due to changes in metabolic abilities or be caused by reduced kidney functioning, including reduced glomerular filtration rate and renal blood flow, known pharmacokinetic changes observed with aging (Mangoni and Jackson, 2004; Wooten, 2012). Analyzing differences in DON metabolism and integrity of kidney functioning with aging will be therefore be an important objective in future studies.

DON concentrations in the brains of aged mice were also elevated at 2 h PI compared with 1 h PI, as seen in the kidney, while the levels of this toxin in brains of young adults decreased from 1 h PI to 2 h PI. Importantly, DON concentrations were still higher in aged mice at 12 h PI. These findings are notable because DON is known to directly affect the brain, including the induction of c-Fos, a strong indicator of neuronal activation (Dragunow and Faull, 1989; Girardet *et al.*, 2011; Bonnet *et al.*, 2012). Further exploration of possible differential effects of DON on the brains of aged mice could provide new insights into why animals are more sensitive than adults to the anorectic effects of DON.

Increased proinflammatory plasma cytokine responses in aged mice compared with adults might be a critical factor for the heightened anorectic responses observed in aged mice. Previous investigations of LPS-induced cytokine responses also reported an increase in proinflammatory cytokine responses in aged animals compared with adults, particularly in plasma IL-6 (Saito *et*

*al.*, 2003; Huang *et al.*, 2008). Some studies have reported increased baseline levels of proinflammatory cytokines in aged mice, a process known as inflammaging (De Martinis *et al.*, 2005; Godbout and Johnson, 2009). Plasma IL-6 was slightly elevated in aged control mice (0.02-0.3 ng/mL) in our studies in our acute i.p. exposure in comparison with adults (0.01-0.02 ng/mL). The baseline levels of plasma IL-6 are likely a response to the i.p. injection. As no elevation in plasma cytokine levels was observed at 12 h PI in control aged mice, elevated plasma cytokine levels in DON-treated aged mice are a result of toxin exposure and not a proinflammatory phenotype prior to exposure. The peak elevation of plasma IL-6 and IL-1β at 2 h PI in aged mice also coincides with the highest kidney concentration of DON. Together these observations suggest that decreased ability to excrete DON is linked to increased proinflammatory cytokine responses, and are highly consistent with their potential to contribute to the increased anorectic response in aged mice.

Elevated PYY and CCK responses in aged mice relative to adults could also elicit increased food refusal in aged mice. These hormones are known to cause satiety and have been previously shown by our lab to increase with exposure to DON (Flannery *et al.*, 2012; Wu *et al.*, 2013; Wu *et al.*, 2014b). Again, increased PYY and CCK could be caused by slower excretion of DON. It is also possible that DON evokes a greater induction of these gut satiety hormones innately, as previous research in humans has shown greater induction of PYY and CCK with food consumption in aged subjects compared with younger individuals (Di Francesco *et al.*, 2005; Moss *et al.*, 2011; Moss *et al.*, 2012). Our findings also support the idea that PYY, and to a lesser extent CCK, are indeed basally elevated in aged animals and exposure to DON elicited a further elevation of these satiety hormones.

The greater suppression of food intake in aged mice than in adults upon dietary DON exposure is consistent with the observation of greater feed refusal in aged animals versus adults in the acute i.p. exposure to DON. Aged mice consuming less food than adult animals also indicates that they are more sensitive to adverse effects of DON. Because aged animals ingest less food, they also consume less DON, suggesting that aged mice have a lower threshold of the amount of toxin that they can tolerate.

At the termination of Study 3, weight loss in aged and adult mice appeared to plateau. It might be speculated that this results from induction by DON of UGT enzymes and/or bacterial ability to convert DON to DOM-1, resulting in a greater capacity to metabolize DON. In a 2 year study assessing chronic exposure of mice to diets containing 1, 5, and 10 ppm DON, body weight gain appeared to be unaffected by DON exposure for the first 100 d of treatment diets (Iverson *et al.*, 1995). After this time point, weight gain in mice on diets containing 5 and 10 ppm began to decrease while the body weight gain of mice on diets containing diets 0 and 1 ppm DON continued to increase until approximately 500 d of treatment diets. The results presented in the 2 year chronic feeding study, along with our findings in Study 3, indicate that while some adaptation to DON does occur, chronic exposure to DON over a lifetime will still result in weight reduction.

## 4. Experimental Section

### *4.1 Animals*

The Institutional Animal Care and Use Committee at Michigan State University approved all animal experiments. Adult male (3 mos) and aged male C57BL6 mice (22 mos) were

purchased from National Institute on Aging colony (Charles River Laboratories, Wilmington, MA, USA). Mice were housed singly in polycarbonate cages with sifted aspen bedding on a 12 h light/dark cycle, with constant temperature (21-24$^0$C) and humidity (40-55%). In all experiments mice were acclimated to high fat pellet diet (45% kcal from fat; Research Diets, Inc., New Brunswick, NJ) 1 wk prior to DON exposure. The high fat diet has previously been determined to provide the most efficient food recovery in acute DON exposure studies (Flannery *et al.*, 2011).

*4.2 DON*

DON used in i.p. injections and experimental diet was obtained from Dr. Tony Durst (University of Ottawa, Canada) and purity was verified to be 98% by elemental analysis **(**Galbraith Labs, Knoxville, TN). For all i.p. injections, DON was dissolved in sterile Dulbecco's phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) to yield 100 µL injection volumes. High fat pellet diets containing 0, 1, 2.5 and 10 ppm DON were formulated by Research Diets, Inc. DON concentration of diets were confirmed using the Veratox high sensitivity (HS) enzyme-linked immunosorbent assay (ELISA; Neogen, Lansing, MI) according to manufacturer protocol.

*4.3 Experimental design*

*4.3.1 Study 1*

Effects of age on food intake after acute i.p. DON exposure were assessed as summarized in Figure 4.1 using a protocol previously described by our laboratory (Flannery *et al.*, 2011). After acclimation, adult and aged mice (n= 5/group) were fasted from 10:00 to 18:00 h, and injected i.p. with either 0 (vehicle), 1 or 5 mg/kg bw DON. Food consumption was measured

hourly 1 to 7 h post exposure, and at 12, 20, 24 and 36 h post injection (PI). Measurements were conducted under red light conditions during dark cycle. We choose to use i.p. exposure for this and the following study to bypass microbial metabolism to DOM-1, a non-toxic metabolite of DON, because rodent gastrointestinal tract microflora are capable of forming this metabolite, while human microflora are typically unable of this type of metabolism (Maresca, 2013).

*4.3.2 Study 2*

Effects of age on DON tissue levels and plasma concentration of proinflammatory cytokines and satiety hormones were measured after acute i.p toxin injection. Adult and aged mice (n = 9-10/group) were fasted as in Study 1 and exposed to 1 mg/kg bw DON in PBS or PBS vehicle via i.p. injection. Mice were euthanized with $CO_2$ at 1, 2, 4, and 12 h PI without food replacement. Blood was collected via cardiac puncture and then kidney, liver, spleen, heart and brain were collected. Plasma was isolated from blood by centrifugation at 3,500 x g for 10 minutes at 4°C. Plasma and organs were stored at -80°C until analysis.

*4.3.3 Study 3*

Effects of age on food intake and body weight changes elicited by dietary DON exposure were assessed. After acclimation, adult and aged mice were randomized into equal weight groups by age group (n= 5-6/group) and placed on high fat diets containing 0, 1, 2.5 and 10 ppm DON. Mice were given 2 weighed pellets (approximately 7 g) to allow for *ad libitum* food consumption. Body weights were measured daily at 10:00 AM for 14 d. Food intake was also measured each day at this time for 2 d. After 2 d, mice on diets containing DON progressively began to shred the pellets into fine particles preventing accurate food recovery. After 14 d of DON exposure, mice were euthanized under $CO_2$ at 8:00 AM. Food access was *ad libitum* prior

to euthanasia. Blood was collected via cardiac puncture and then kidney, liver, spleen, heart and brain collected. Plasma was isolated from blood. Plasma and organs were stored at -80°C until analysis.

### *4.4 Analytical*

#### *4.4.1 DON analysis*

DON quantification in plasma and organs in Study 2 were analyzed using the Veratox high sensitivity (HS) ELISA (Neogen, Lansing, MI) as described by Pestka *et al.* (2008) with slight modifications. Briefly, organs were homogenized 1:1 in PBS (except the heart, which was homogenized 1:2). Tissue homogenates were heated at 100°C for 5 min and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting supernatant was analyzed by ELISA. Chromogenic endproduct was measured using an F3 ELISA plate reader at 650 nm and Softmax software (Molecular Devices, Menlo Park, CA). As previously described by our lab (Clark *et al.*, 2015b), DON was reported as DON equivalents because the ELISA was found to be 100% cross reactive with DON3GlcA, a major metabolite of the toxin produced in rodents,

#### *4.4.2 Proinflammatory cytokine analyses*

Plasma levels of the proinflammatory cytokines IL-6, IL-1β, and TNF-α were determined in Study 2 and 3 using Duoset ELISAs from R&D Systems (Minneapolis, MN). Due to limited plasma quantities, equal volumes of mouse plasma samples from Study 2 were pooled within groups and ran in technical replicates (n = 4 rep).

*4.4.3 Satiety hormone analyses*

Plasma levels of the gut satiety hormones CCK and PYY3–36 were analyzed using ELISA kits for CCK (CCK [26–33], nonsulfated; human-, rat-, and mouse-specific) and PYY (PYY [3–36]; mouse-, rat-, porcine-, and canine-specific; Phoenix Pharmaceuticals, Burlingame, CA) in with plasma samples from Study 2. CCK and PYY were not measured in Study 3 because animals had *ad libitum* access to food prior to study termination.

*4.5 Statistical analysis*

Statistical analysis was conducted by SigmaPlot version 11.0 (Jandel Scientific; San Rafael, CA). Statistical comparisons between ages were made at each time point using a Student's t-test, unless normality failed. If normality was not met, a Mann-Whitney Rank Sum test was performed. Statistical comparisons between age and dose were made at each time point using a one-way analysis of variance (ANOVA), unless normality failed. A Kruskal–Wallis one-way analysis of variance by ranks was performed if normality was not met. Student–Neuman–Keuls was used in all post-hoc analysis for parametric and non-parametric animal groups of equal numbers. Dunn's test was used in post-hoc analysis of non-parametric analysis of unequal animal group numbers. Pearson product moment correlations were performed to determine statistical significance between food consumption and treatment levels of DON diets by age and day in Study 3. Differences were considered significant when $p < 0.05$.

## 5. Conclusions

The results presented herein indicate that aged mice are more susceptible than their young adult counterparts to DON-induced anorexia following either acute i.p. or dietary DON

exposure. In addition to reduced food intake in dietary exposure studies, a greater suppression of body weight gain was seen in aged mice compared with adult mice. Aged mice exhibited slower tissue clearance of DON and increased proinflammatory plasma cytokine and gut satiety hormone responses compared with adult mice after acute i.p. exposure to DON. Collectively, these findings suggest that advanced life stage should be considered when formulating risk assessments for DON and other trichothecene mycotoxins.

## CHAPTER 4: Effects of Sex and Age on Murine Deoxynivalenol Metabolism

Data in this chapter will be included in a manuscript: Clark, E.S., Amuzie, C.J., Berthiller, F., and Pestka, J.J.


### Abstract

Deoxynivalenol (DON, vomitoxin) excretion profiles in urine and feces of C57BL6 mice were compared by sex and age. Males and aged mice were found to have slower urinary DON excretion in comparison with female and adult mice, respectively. In contrast, males and aged mice had greater excretion of the total DON dose in feces than female and adult mice, though fecal DON recovery accounted for only a small percentage of the total DON dose recovered. Hepatic DON glucuronidation activity was found to be species-specific, though sex and age differences were not consistently present. Hepatic UDP-glucuronic acid (UDP-GA) concentrations did not differ by sex or age in mice. Renal DON glucuronidation activity by either mouse or mink microsomes was undetectable.


### 1. Introduction

Deoxynivalenol (DON, vomitoxin), is a type B trichothecene mycotoxin produced by *Fusarium graminearum* that frequently contaminates cereal crops including wheat, corn, and barely (Wu *et al.*, 2014a). This mycotoxin is of public health concern because it targets the gastrointestinal and innate immune systems. In animals capable of emesis, DON rapidly causes vomiting (Forsyth *et al.*, 1977; Wu *et al.*, 2013). Mice however, are incapable of vomiting and instead show feed refusal (Prelusky *et al.*, 1997; Flannery *et al.*, 2011). Previous research in our lab has reported greater sensitivity to DON-induced feed refusal in male mice compared to

female mice and aged mice compared to adult mice (Clark *et al.*, 2015a; Clark *et al.*, 2015b). It was hypothesized that those different sex- and age-dependent anorectic responses to DON were the result of slower clearance and metabolism of the toxin. However, DON metabolism has not been studied in mice and research comparing DON excretion profiles and metabolism by sex and age in other species is limited. One study that did compare sex differences of DON excretion in rats, found that male rats orally exposed to 0.5 and 2.5 mg/kg bw DON excreted 30% and 18% less of the total cumulative in urine, respectively, than female rats (Wan *et al.*, 2014).

Metabolism and excretion of DON is highly-species dependent (Wu *et al.*, 2010; Maresca, 2013). A de-epoxide derivative of DON (9,12-diene DON; DOM-1) was the first metabolite identified and is the product of GI tract bacterial metabolism (Yoshizawa *et al.*, 1983; Worrell *et al.*, 1989). Polygastric animals (i.e. ruminates) and poultry have a high concentration of microflora present both before (i.e. croup, rumen) and after the small intestine, where DON is absorbed. Thus they are better able to produce DOM-1 than monogastric animals such as swine, rodents and humans (Smith, 1965; Avantaggiato *et al.*, 2004; Maresca, 2013). DOM-1 formation in humans is reported to occur at low or undetectable rates (Lattanzio *et al.*, 2011). One study found that only one of five human fecal samples incubated with DON was able to form DOM-1 (Gratz *et al.*, 2013).

Glucuronidation of DON is another widely studied form of metabolism. Formation of DON-3 glucuronic acid (DON3GlcA) is the dominant glucuronide formed by non-human animals with DON-7 glucuronic acid (DON7GlcA) being a minor glucuronide metabolite (Maul *et al.*, 2012; Maul *et al.*, 2015). An exception to this profile of glucuronide formation is the pig, which forms DON-15 glucuronic acid (DON15GlcA) as a minor metabolite instead of DON7GlcA. In comparison, humans produce DON15GlcA as the major glucuronide of DON

with DON3GlcA being a minor glucuronide metabolite (Sarkanj *et al.*, 2013; Warth *et al.*, 2013; Maul *et al.*, 2015).

Recent studies have reported that DON sulfonates also result from DON metabolism. Wan et al (2014) found that 24% of the total DON dose was excreted as a yet uncharacterized DON sulfonate in rats orally exposed to 0.5 and 2.5 mg/kg bw to the toxin (Wan *et al.*, 2014). In the same study, production of DON sulfonate metabolite was even higher in poultry orally exposed to 2.5 mg/kg bw of the mycotoxin, with 89% of the total DON dose excreted as the tentatively named DON-3α-sulfate. Another recent report of DON sulfonate metabolite production by rats after oral exposure to DON, found that 48% of the total DON dose was excreted as sulfonated DON metabolites (Schwartz-Zimmermann *et al.*, 2014a).

The aims of this study were to compare DON excretion profiles *in vivo* and the capacity to glucuronidate DON *ex vivo* by sex and age in mice. *Ex vivo* studies also investigated species differences in DON glucuronidation in mink and humans. The results presented in this chapter indicate that male mice and aged mice excrete DON more slowly than female and adult mice, respectively, and that differences in excretion do not appear to be linked to reduced capacity to glucuronidate DON. Hepatic glucuronidation did not differ consistently by sex or age, but did differ by species. DON glucuronide formation was undetectable in renal microsomes isolated from either mouse or mink.

## 2. Materials and Methods

### *2.1 Animals*

The Institutional Animal Care and Use Committee at Michigan State University approved all animal experiments prior to their initiation. For comparison of sex differences, adult male and

adult female C57BL6 mice (12 wk old) were purchased from Charles River Laboratories (Portage, MI). For comparison of age differences, adult male (3 mos old) and aged male C57BL6 mice (22 mos old) were purchased from National Institute on Aging colony (Charles River Laboratories, Wilmington, MA, USA). Mice were housed singly under a 12 h light/dark cycle, with constant temperature (21-24°C) and humidity (40-55%). Mice were housed in polycarbonate cages with aspen bedding in all experiments, except when metabolism cages were used. In the experiments involving metabolism cages, mice were rested 1 wk after arrival in polycarbonate cages and then transferred to metabolic mouse cages (Techinplast, West Chester, PA). All mice were acclimated to high fat pellet diet (45% kcal from fat; Research Diets, Inc., New Brunswick, NJ) for 1 wk prior to DON exposure or sacrifice to maintain consistency among experiments. High fat diet was verified to be free of DON using a DON Veratox HS (high sensitivity) ELISA (Neogen, Lansing, MI) according to manufacturer's protocol. Male and female American mink (*Neovison vison*; 1-2 yrs old) were obtained from the Michigan State University (MSU) Experimental Fur Farm. Housing conditions met those specified in the Standard Guidelines for the Operation of Mink Farms in the United States (USA, 2010) . Mink organs were obtained from separate study using male-female mink pairings. Mink were housed in pairs in wire cages (61x76x46 cm) and provided with a nest box (31x25x25 cm) with aspen shavings of excelsior (wood wool) within a pole barn. Mink were housed under a 14 h light/10 h dark cycle with temperature and humidity dependent on the ambient environment. Mink were euthanized individually in a transparent plastic container, into which 10 ml of isoflurane was sprayed. Animals were rendered unconscious in less than 60 s with no obvious signs of stress or suffering. The container was then filled with $CO_2$ for three minutes to ensure death.

*2.2 Chemicals and reagents*

DON used for i.p. injections and microsomal incubations was obtained from Dr. Tony Durst (University of Ottawa, Canada) and purity was verified to be ≥ 98% by elemental analysis (Galbraith Labs, Knoxville, TN). Uridine 5′-diphosphoglucuronic acid (UDP-GA) and alamethicin (from *Trichoderma viride*) were purchased from Sigma-Aldrich (St. Louis, MO). 3-hydroxybenzo[a]pyrene was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Hepatic and renal microsomes from mouse (n=6/gp) and mink (n=8/gp) were prepared according to a previous described method (Lake, 1987). Microsomal protein content was determined using the Pierce BCA Protein Assay kit (Lifetchnologies; Grand Island, NY). Male and female human liver microsomes (pool of 10 individuals each) and male Hartley albino guinea pig liver microsomes were obtained from Xenotech (Lenexa, KS). Aged human liver microsomes (mixed gender, pool of 10 individual > 75 yrs) were purchased from Lifetechnologies (Grand Island, NY).

*2.3 Determination of urinary and fecal DON excretion*

Effects of sex and age on urinary and fecal excretion of DON to exposure of 1 mg/kg bw DON via intraperitoneal (i.p.) injection were compared using Nalgene metabolic cages (MTB-0311; Nunc-Nalgene, Rochester, NY). After acclimation to metabolism cages for 1 wk, mice (n = 8/gp) were fasted from 10:00 AM to 6:00 PM, exposed to DON, and food replaced. DON was dissolved in sterile saline to equal 1 mL injections based on preliminary studies revealing that this injection volume was found to facilitate early urine collection time points. Urine and fecal sample collection tubes were kept on ice during the duration of the experiment. Urine samples were collected at 2, 4, 8, 12, and 24 h post injection (PI) by thoroughly rinsing collection surface with 3 mL distilled water. This process was repeated 3 times with the same 3 mL of water at

each timepoint. Fecal samples were collected at 4, 8, 12, and 24 h PI. Fecal samples were not collected at 2 h PI due to variable and low sample amounts. Samples were stored at -80°C immediately after collection until analysis.

Feces were suspended in PBS (1:5, w/v) for 10 min at room temperature and then homogenized. Urine and fecal homogenates were then centrifuged at 15,000 x g for 10 min at 4°C. Supernatant was removed and then heated at 100°C for 5 min. Sample were centrifuged again at 15,000 x g for 10 min at 4°C and resulting supernatant was used for analysis. Urinary and fecal DON equivalent concentrations were measured using a DON Veratox HS ELISA with modifications as previously described (Amuzie *et al.*, 2010). DON was reported as DON equivalents because this ELISA was 100% cross-reactive with DON3GlcA, the major glucuronide DON metabolite in the mouse (Clark *et al.*, 2015b). Cross-reactivity with other DON glucuronides or sulfonates was not determine because of lack of available standards.

*2.4 Hepatic and renal microsomal conversion of DON to DON glucuronides*

Effects of sex, age, and species on DON metabolism by microsomal enzymes were compared. Conditions of microsomal incubations were as previously reported (Maul *et al.*, 2015). Reaction mixtures (200 μL) contained: 100 mM potassium phosphate buffer (pH 7.4); 5 mM $MgCl_2$; 2.5 mM UDP-GA; 25 μg/mL alamethicin; 1 mg/mL microsomal protein; and 4 μL of either 5 μM or 20 μM DON in methanol/water 50/50 (v/v). Reactions were preincubated at 37°C for 5 min. UDP-GA was then added to initiate reaction and tubes further incubated at 37°C for 60 min. The addition of 200 μL ice-cold acetonitrile terminated the reaction and tubes were centrifuged at 14,000 x g for 5 min. A supernatant aliquot (200 μL) was then evaporated to dryness in a vacuum concentrator. Samples were analyzed in triplicate by LC-MS/MS using an AB SciexQTrap 5500 MS system (Foster City, CA) with a Turbo V electrospray ionization (ESI)

source combined with an Agilent 1290 series LC system (Waldbronn, Germany) with LC-MS/MS conditions as previously described (Maul *et al.*, 2015).

*2.5 Determination of hepatic UDP-GA concentrations*

Hepatic UDP-GA concentrations were determined by UDP-GA dependent formation of 3-hydroxybenzo[a]pyrene glucuronide as described in a prior study (Singh *et al.*, 1980). Mice (n=10/gp) were fasted from 10:00 AM to 6:00 PM as in previous studies comparing sex and age differences in tissue DON concentrations. Mice were humanely euthanized by anesthetization with isoflurane and then cervical dislocation. Small pieces of liver (approx. 50 mg) were quickly excised and immediately dropped into 5 mL of boiling distilled water for 3 min. Tissues were cooled on ice and then dounce homogenized in sterile water. Homogenized samples were centrifuged at 3000 x g for 10 min at 4°C. Supernatant was removed and stored at -80°C until analysis. The reaction mixture (200 μL) contained: 20 μmol Tris buffer (pH 7.6); 1 μmol $MgCl_2$; 0.01% Brij-58 (w/v); 10 nmol of 3-hydroxybenzo[a]pyrene in 10 μL of methanol; 50 μL liver tissue homogenate; and 50 μg of guinea-pig liver microsomal protein. Reaction tubes were held on ice until incubation at 37°C in a water bath with mild shaking for 30 minutes. To terminate reaction, reaction mixtures were added to 6 mL of chloroform/methanol (2:1, v/v) and and 0.8 mL of distilled water and vigorously shaken. Tubes were centrifuged at 1000 x g for 1 min. 200 μL of the aqueous-methanol phase was added to black 96-well plates. Fluorescence of benzo[a]pyrene glucuronide endproduct was measured at 378 nm excitation/425 nm emission. UDP-GA amounts were determined from a standard curve of known UDP-GA concentrations. Samples were analyzed in duplicates.

*2.6 Statistical analysis*

All statistical analyses were conducted using SigmaPlot version 11.0 (Jandel Scientific; San Rafael, CA). Statistical comparisons by sex and by age at a specific time point were made by a Student's t-test, unless normality failed in which case a Mann-Whitney Rank Sum test was performed. A one-way analysis of variance (ANOVA) was used to determine statistical significance between sex and age in experiment comparing DON glucuronidation by human liver microsomes. Differences were considered significant when $p < 0.05$.

## 3. Results

*3.1 Total DON excretion is slower in adult male than adult female mice*

DON excretion in urine and feces by male and female mice was compared over 24 h PI following exposure to 1 mg/kg bw DON. Males exhibited slower clearance rates of DON than females (Figure 4.1). At 2 h PI, female mice had excreted 22% of the DON dose while males had excreted only 14%. By 8 h PI, the majority of the recoverable dose had been excreted in the urine of males and females, accounting for 49% and 60% of the total dose, respectively. Interestingly, 8 h to 24 h PI, male mice excreted significantly more of the cumulative DON dose in the feces than females, though this only accounted for a small percent of the total recovered dose. Upon study termination (24 h PI), 63% of the total dose had been recovered in female excreta (62% in urine, 1.3% in feces) and 52% of the total dose had been recovered in male excreta (49% in urine, 2.6% in feces).

*3.2 Aged mice excrete DON more slowly than adult mice*

DON excretion in urine and feces by aged and adult mice were compared over 24 h PI when exposed to 1 mg/kg bw DON. Aged mice had slower clearance rates of DON than adult mice (Figure 4.2). At 4 h PI, adult mice had excreted 66% of the total DON dose. Aged mice at this time point had only excreted 25% of the total DON dose. Aged mice excreted significantly less of the total DON dose in the urine at 4 h PI than adults and at all other time points in urinary excretion comparisons. By 8 h PI, adult mice had excreted 80% of the total dose and only 3% of the dose was recovered between 8 to 24 h with a total of 82% of the dose recovered in the urine and 1.1% in feces. In comparison, aged mice had only excreted 51% of the total dose at 8 h PI and excreted another 8.5% of the dose between 8 to 24 h PI. This accounted for 57% of the total dose recovered in the urine and 2.5% in the feces of aged animals at 24 h PI. It is of interest is that aged mice showed a trend toward excreting more of the toxin in the feces than adults and this difference was significant at 24 h PI.

*3.3 Hepatic microsomal glucuronidation of DON differs by sex, age, and species*

The effects of sex, age and species on DON glucuronidation by microsomal enzymes were compared (Table 4.1). In mouse microsomes treated with 5 μM DON with DON3GlcA formation was higher in males than females and higher in aged than adult mice. Similar trends were observed for microsomes treated with 20 μM DON.

Differences in DON glucuronides formed and glucuronidation rates were apparent between human and non-human animals. In liver microsomes from mice and mink, only formation of the DON3GlcA was detectable. Hepatic microsomes of humans produced both the DON15GlcA and DON3GlcA when treated with 20 μM DON, with the DON15GlcA form being

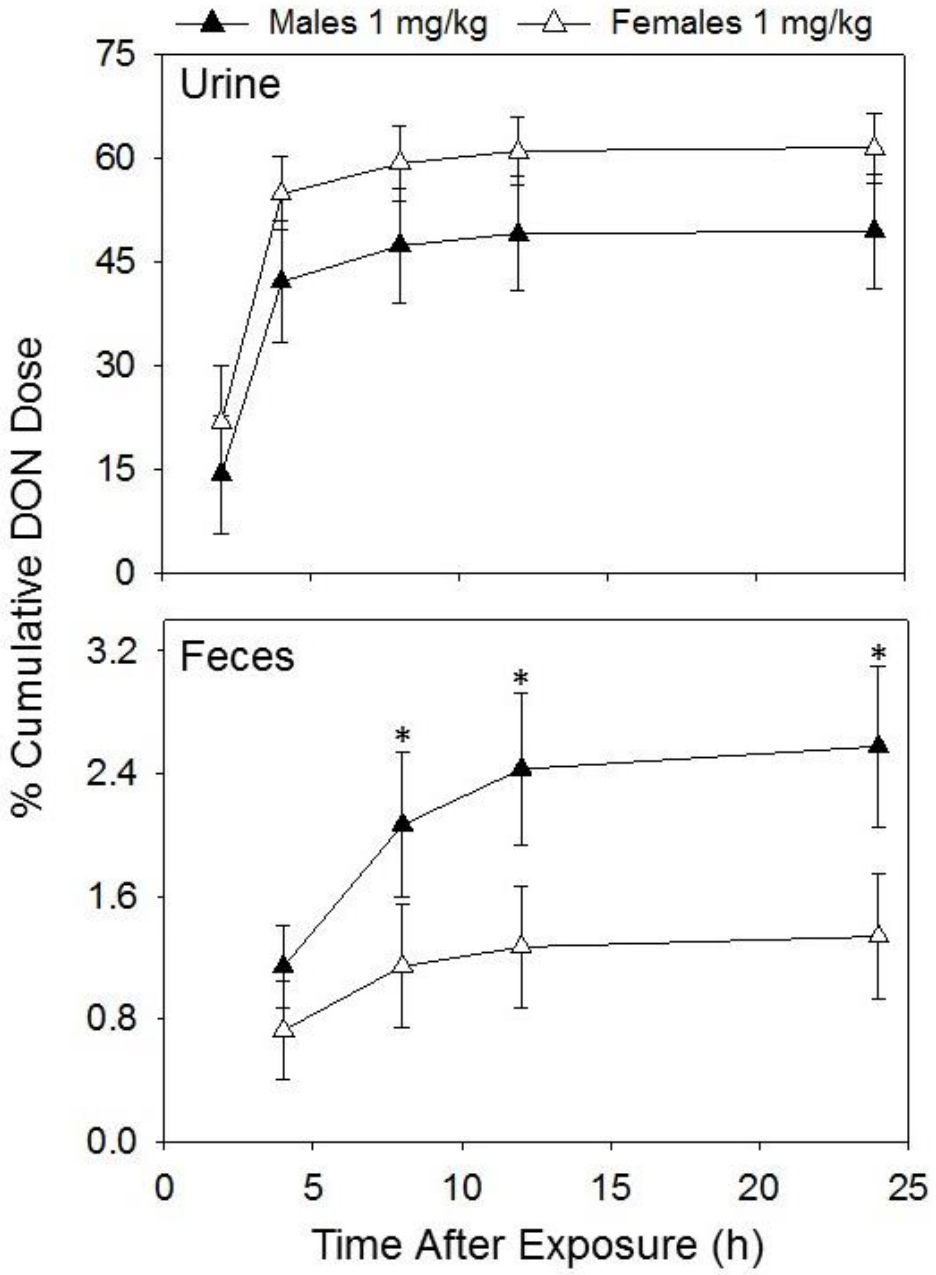


Figure 4.1 Total urinary and fecal DON excretion is slower in male mice than female mice. Data are mean $\pm$ SEM (n = 8/gp). Asterisks indicates statistical significance from female at time point ($p < 0.05$).

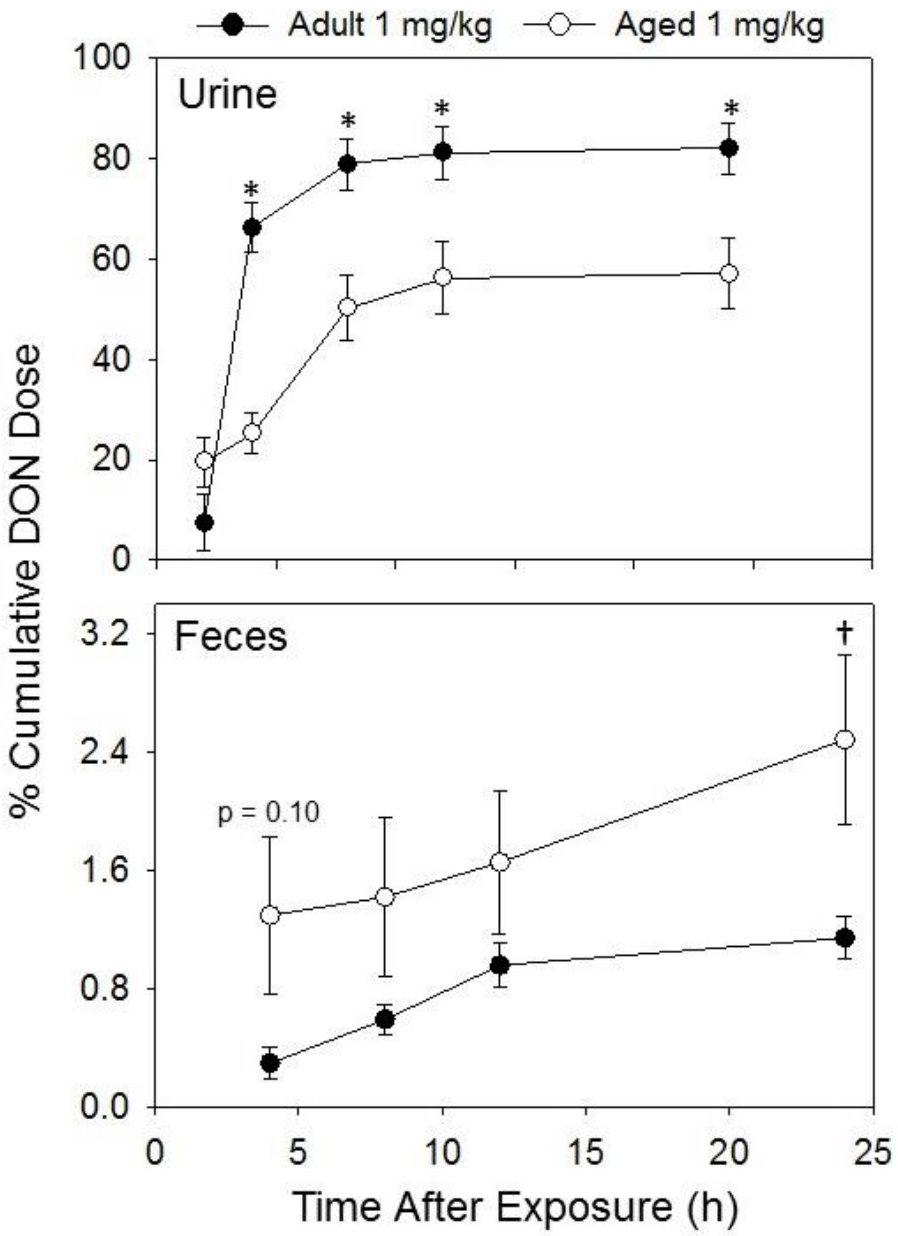


Figure 4.2 Total urinary and fecal DON excretion is slower in aged mice than adult mice. Data are mean ± SEM (n = 8/gp). Asterisks indicates statistical significance from aged at time point and dagger indicates statistical significant from adult at time point ($p < 0.05$).

the predominant glucuronide formed. When human liver microsomes were treated with 5 μM DON, only formation of the DON15GlcA was detectable.

Incubations with either concentration of DON with renal mouse and mink microsomes did not produce detectable DON glucuronide metabolites.

*3.4 Hepatic UDP-GA not affected by age or sex in the mouse*

When the concentrations of UPD-GA were measured in the livers of mice, no sex or age differences were observed (Figure 4.3). The concentrations reported here are in agreement with the UDP-GA levels previously reported in mice (Howell and Klaassen, 1991).

## 4. Discussion

This is the first report of DON excretion profiles in mice, as only rats have previously been used to study rodent metabolism of DON (Maresca, 2013). Comparison of sex and age excretion profiles were made based on previous studies from our lab identifying increased sensitivity to DON-induced anorexia in male mice compared to female mice and aged mice compared to adult mice (Clark *et al.*, 2015a; Clark *et al.*, 2015b). First, it was found that urinary and fecal excretion of DON varied by sex and age. Secondly, we found that this variation was unlikely caused by a reduced capacity to glucuronidate DON. As higher tissue concentrations of DON equivalents were reported in groups more sensitive to DON-induced anorexia, it is possible that male mice and aged mice have a reduced capacity to clear the toxin than female and adult mice. Thirdly, DON treatment of renal microsomes from mice and mink did not yield detectable levels of DON glucuronides indicating that these metabolites are not formed in the kidney.

Table 4.1 Species specific glucuronidation of DON by liver microsomes.

| | DON Glucuronide Formation (pmol/min/mg protein) | | | |
|---|---|---|---|---|
| | 5 µM DON | | 20 µM DON | |
| **Species of Microsome** | **DON3GlcA** | **DON15GlcA** | **DON3GlcA** | **DON15GlcA** |
| Male mouse | 240 ± 18 | ND | 970 ± 127 | ND |
| Female mouse | 140 ± 15* | ND | 600 ± 48 | ND |
| Adult mouse | 250 ± 3 | ND | 920 ± 83 | ND |
| Aged mouse | 210 ± 10† | ND | 810 ± 24 | ND |
| Male mink | 260 ± 9 | ND | 1100 ± 22 | ND |
| Female mink | 230 ± 10 | ND | 1000 ± 2 | ND |
| Male human | ND | 30 ± 1 | 40 ± 6 | 120 ± 3 |
| Female human | ND | 30 ± 2 | 50 ± 1 | 120 ± 4 |
| Aged human | ND | 30 ± 2 | 40 ± 3 | 130 ± 2 |

Data are mean ± SEM (n=3/rep). ND indicates not detected. Asterisk indicates significance from male at treatment level and dagger indicates significance from adult at treatment level ($p < 0.05$).

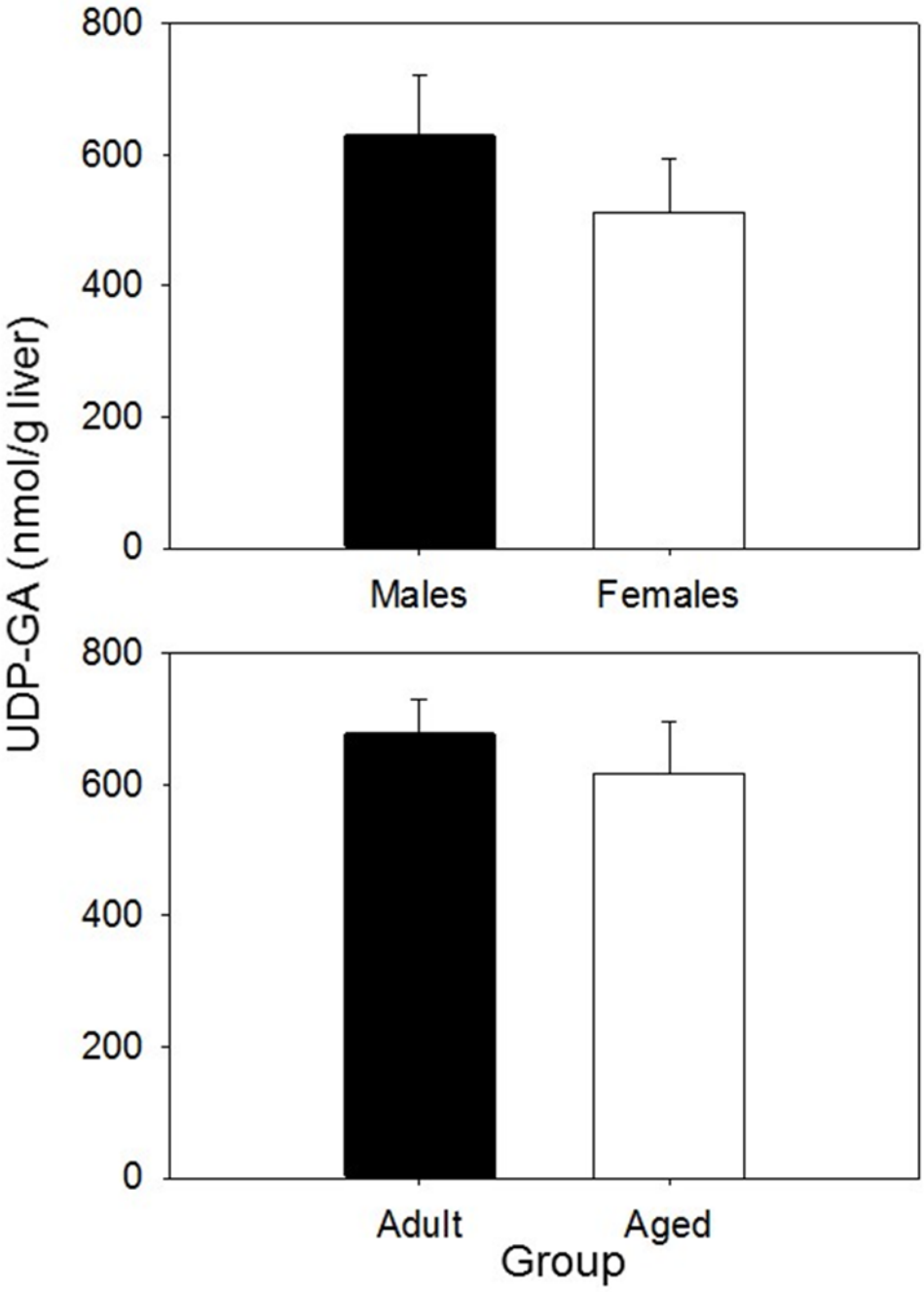


Figure 4.3 Hepatic UDP-GA concentrations do not differ by sex or age. Mice from microsomal DON treatments and hepatic UDP-GA were from different sources as different processing techniques were required. Data are mean $\pm$ SEM (n = 10/gp).

A previous comparison of DON excretion by sex in rats also reported lower recovery of DON and DON metabolites in urine of male rats compared with female rats (Wan *et al.*, 2014). When orally gavaged with 0.5 mg/kg bw DON, 57% of the total DON dose was eliminated in the urine of female rats, while only 27% of the total DON dose was recovered in the urine of similarly treated male rats. This difference was also present when rats were gavaged with 2.5 mg/kg bw DON with female rats and male rats excreting 42% and 24%, respectively, of the total DON does via urinary elimination. In our study, male mice excreted 11% less of the total DON dose in the urine than females over the duration of the study. One possible explanation for slower urinary DON excretion in males than females is a previous report that female C57BL6 mice innately drink more water and produce more urine than males when adjusted by body weight (Stechman *et al.*, 2010). Thus, females may excrete DON more rapidly due to higher urine production.

In these excretion studies in mice, i.p. exposure was chosen for two reasons: 1) i.p. exposure of DON is often used in risk assessment studies and 2) as an attempt to bypass metabolism by GI microflora to DOM-1. Because i.p. exposure was used in studies comparing sex and age differences to acute DON exposure, we wanted to determine what excretion profiles were occurring under similar conditions. Acute i.p. DON exposure was chosen in previous studies comparing sex and age differences to try to more closely mimic human metabolism. Human GI tract microbiota are generally incapable of metabolizing DON to DOM-1, while rodent microflora is capable of this type of metabolism (Eriksen and Pettersson, 2003; Turner *et al.*, 2011; Gratz *et al.*, 2013; Maresca, 2013).

Our findings were consistent with other studies suggesting DON metabolism is also species dependent (Wu *et al.*, 2010; Maresca, 2013). DON3GlcA is the major DON glucuronide

metabolite of non-human animals found in urine (Maresca, 2013). DON3GlcA is also shown to be the predominant glucuronide formed by non-human animal hepatic microsomes treated with DON, while the formation of DON7GlcA is slight in comparison (Maul *et al.*, 2012; Maul *et al.*, 2015). An exception to this pattern of glucuronide formation in non-human animals is porcine hepatic microsomes, which do not form DON7GlcA but instead form DON15GlcA as a minor metabolite. In humans, DON15GlcA is the major glucuronide metabolism formed, with DON3GlcA being a minor metabolite found in both urine samples and *ex vivo* microsomal treatments with DON (Sarkanj *et al.*, 2013; Warth *et al.*, 2013). Our results comparing mouse, mink and human hepatic microsomal glucuronidation of DON are consistent with previous reports of DON glucuronide formation, with the exception of DON7GlcA not being detected in any species. Additionally, while rates of DON glucuronide formation in hepatic microsomal treatments were similar in mice and mink, human microsomal treatments yielded lower rates. This is also consistent with what has been reported in the literature (Maul *et al.*, 2012; Maul *et al.*, 2015).

Formation of other DON metabolites could possibly contribute to differences in excretion profiles by sex and age. Bacterial formation of DOM-1 and DON glucuronides have been recognized as the major end-products of DON metabolism. However, two recent studies conducted during this dissertation have reported that sulfonation of DON is likely to be another important type of DON metabolism in rats and poultry (Schwartz-Zimmermann *et al.*, 2014b; Wan *et al.*, 2014). Wan et. al (2014) found that after oral exposure, 89% of the total DON dose was recovered in poultry excreta as a newly identified metabolite, DON-3α-sulfonate (Wan *et al.*, 2014). This study also reported that 24% of the total DON dose was recovered as a yet not characterized DON sulfonate in rat excreta when orally exposed to the toxin. Schartz-

Zimmerman et al. (2015) provided further characterization of the DON sulfonates and reported that with oral toxin exposure in rats, 48% of the total DON dose was excreted as DON- and DOM-sulfonates, with DONS 2 and DOMS 2 being the major sulfonates formed (Schwartz-Zimmermann *et al.*, 2014b).

Differential formation of the DON sulfonates by sex and age could be a contributing factor to slower excretion rates observed in males and aged mice compared to female and adult mice. Previous studies show evidence that mRNA levels of some sulfotransferases (Sults) are higher in livers of females C57BL6 mice than male (Kocarek *et al.*, 2008; Fu *et al.*, 2012). While mRNA expression of Sults generally increase with age, Sult3a1 and Sult5a1 are have been shown to decrease in the livers of both male and female C57BL6 mice with aging (Fu *et al.*, 2012). DON sulfonates appear later in excretion profiles (detected in feces only after 24 h post exposure), while DON3GlcA was detected in urine up to 6 h PI (Wan *et al.*, 2014).

Metabolism of DON to DON and DOM sulfonates could also contribute to the differences seen in total recovery rates. It should also be noted that DON was administered by oral gavage in the aforementioned studies measuring DON sulfonate formation. This could affect the formation of sulfonates in our studies. While DOM-1 has been reported to have greater cross-reactivity than DON measured by the DON Veratox ELISA, the ability of the ELISA to detect DOM-glucuronides and -sulfonates is unknown and could contribute to differences in excretion profiles and recovery rates (Tangni *et al.*, 2010). Thus, unknown capacity of the ELISA used for DON quantification to detect all metabolites present in excreta is a major limitation of this study. LC-MS/MS analysis of samples should be pursued in the future to resolve possible differential DON metabolite formation by sex and age

Slower urinary excretion in males and aged mice might be the result of lower capacity to transport conjugated metabolites in the kidney and liver. Sex and age differences have previously been reported in hepatic multiple resistance-associated protein (Mrp), one class of transporters responsible for excretion of conjugated compounds (i.e. glucuronides, sulfonates) into the blood and bile (Zhang *et al.*, 2010; Fu *et al.*, 2012). Males and aged mice were found to have lower hepatic mRNA levels for Mrp3 and Mrp4 than females and adults. Mrp3 and Mrp4 are responsible for transportation across the canalicular membrane into bile to be followed by fecal elimination. The hepatic mRNA expression of Mrp2, responsible for transport across the sinusoidal membrane into blood for urinary elimination, did not differ by sex or age. The same pattern of renal mRNA Mrp expression is seen by sex (Maher *et al.*, 2005). While age differences in renal Mrp expression are not available in mice, previous research has shown that mRNA levels of renal and hepatic Mrps are highly correlated in rats (Lu and Klaassen, 2008). Since the transporter responsible for urinary elimination of conjugates did not differ by sex or age, it is unlikely that slower urinary elimination in males compared with females and aged mice compared to adults is the result of differential transport by Mrp.

Age related decreases in kidney functioning could be another contributing slower excretion of DON and DON metabolites in aged mice compared with adults. It is well documented that renal function declines with age (Wooten, 2012; Bolignano *et al.*, 2014). These changes include:  decreased GFR, impaired ability to repair kidney damage, decreased ability to concentrate and dilute urine as necessary, and decreased blood flow to the kidney. A study comparing 2, 12, and 24 mos old C57BL6 male mice found that mice aged 24 mos had significantly increased tubulointerstiatial fibrosis than 2 and 12 mos old mice (Lim *et al.*, 2012). That investigation also found that mice aged 24 mos had significantly increased infiltration of

inflammatory cells than 2 and 12 mos old mice and a greater number of TUNEL-positive cells in the cortical tubular areas and glomerulus than 2 mos old mice. Inclusion and assessment of kidney function in future studies will be critical in future exploration of the effects of life stage to DON exposure.

Fecal elimination was seen by both sex and age to be higher in groups that showed slower urinary elimination, though recovery in feces accounts for only a small portion of the total adminstered dose. This could indicate that greater enterohepatic cycling is occurring in males and aged mice when compared to female and adult, which would prolong toxin elimination in these groups. While increases in mRNA levels of Mrp transporters favoring fecal elimination have not been reported in males and aged mice, an increase in enterohepatic cycling could be a likely explanation of higher fecal excretion in these groups.

**Conclusion**

The results presented in this chapter indicate that sex and aged differences exist in the ability to rapidly excrete DON after i.p. exposure. These results are consistent with previous work in our lab comparing DON-induced anorexia that identified a higher sensitivity to DON in male mice than females and in aged mice than adults. The current study also provides important insight on DON excretion profiles in mice exposed to the toxin with i.p. injection. Future studies should also examine DON metabolite excretion profiles in mice after oral DON exposure to verify that mice exhibit similar patterns of excretion as rats orally exposed to DON.

## CHAPTER 5: Conclusions and Future Directions

DON is known to produce anorectic and emetic behavior in experimental animals, including mice, mink, and pigs. These gastrointestinal effects of DON have been linked to induction of proinflammatory cytokine and satiety hormone responses. Previous studies have to a limited extent investigated sex differences and susceptibility of young animals to DON-induced anorexia. However, the effects of DON on aged animals is largely unaddressed. The research presented in this dissertation provided a more extensive characterization of sex differences to DON-induced anorexia and further examined the toxin's effects in advanced life stage mice.

Sex-dependent responses to DON-induced anorexia were compared in Chapter 2. Male mice were found to be more sensitive to anorexia induction after both acute and dietary DON exposure than females. These effects corresponded to greater suppression of weight gain in males fed DON containing diets. When acutely exposed to DON, males again exhibited slower tissue clearance of the toxin and greater induction of plasma IL-6 than female mice. Higher elevation in this anorectic cytokine and slower tissue DON clearance could contributing factors to male sensitivity to acute DON-induced anorexia.

When the effects of age on DON-induced anorexia were compared in Chapter 3, aged mice were found to be more susceptible to the anorectic effects of DON than adults following either acute i.p. or dietary DON exposure. In addition to reduced food intake in dietary exposure studies, a greater suppression of body weight gain was seen in aged mice compared with adult mice. In acute DON exposure studies, aged mice exhibited slower tissue clearance of DON and increased proinflammatory plasma cytokine and satiety hormone responses compared with adult mice. Induction of plasma proinflammatory cytokine and satiety hormone responses along with

delayed tissue clearance are likely contributing factors to increased sensitivity to DON-induced anorexia in aged mice when compared with adults.

Sex and age comparisons of urinary and fecal DON excretion were also determined (Chapter 4). Male and aged mice had slower urinary excretion of DON than female and adult mice, respectively. It was hypothesized this was the result of sex and age differences in ability to glucuronidate DON. However, *ex vivo* studies comparing hepatic glucuronidation activity of DON did not support this conclusion, nor did differences exist in hepatic levels of UDPGA. It is possible that other differences in metabolic capabilities exist (i.e. sulfonation, microbial metabolism) and these types of DON metabolism should be addressed in future studies.

A major limitation of this study was using the Veratox HS DON ELISA to quantify DON excretion in mouse urine and fecal samples. DON3GlcA was determined to be completely cross-reactive with the ELISA and DOM-1 was previously reported to have greater affinity to the ELISA than DON. Cross-reactivity of other DON metabolites was not determined due to lack of available standards. As detection of other DON metabolites is unknown, this could greatly affect recovery rates and excretion profiles presented in this dissertation. This issue is currently being addressed in our collaborator's laboratory by LC-MS/MS analysis of urine and fecal samples for DON and its metabolites.

Additionally, hepatic glucuronidation of DON was assessed in mink and humans. DON glucuronidation activity did not differ by sex in either mink or humans, nor did toxin glucuronidation differ by age in humans. Primary species differences in glucuronide formation however were observed. Hepatic microsomes from non-human animals (mice and mink) formed only DON3GlcA, while human hepatic microsomes dominantly formed DON15GlcA and

DON3GlcA was a minor metabolite. Renal microsomes from mice or mink did not produce detectable DON glucuronide metabolites.

Based on the discoveries in this dissertation, future research directions should consider the following:

1. While age related changes to DON-induced feed refusal were compared in male mice, it is unknown if aged female mice would present similar increases in anorectic responses relative to adult female mice. Future research should explore if sex dependent responses to DON are present throughout life stages.
2. It is possible suboptimal kidney functioning could contribute to increased sensitivity to DON in aged mice (Chapter 3 and 4). Measures of kidney health and function should be related to increased DON sensitivity in future studies. Exploring the effects of kidney injury on DON-induced anorexia could also provide important insight on risk assessment.
3. Excretion profiles of DON and specific DON metabolites in urine and feces of mice after oral exposure to DON needs to be examined and compared to excretion profiles observed following i.p. DON exposure.

The results presented in this dissertation indicate that anorectic responses to DON exposure have the potential vary due to individual characteristics, including sex and age. Therefore, sex and advanced life stage should be considered when formulating risk assessments for DON and other trichothecene mycotoxins.

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