Rainbow Trout (Oncorhynchus mykiss) as a Sensitive Animal Model for Investigating the Effects of the Fusarium Mycotoxin Deoxynivalenol (DON)

by

Jamie Marie Hooft

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RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AS A SENSITIVE ANIMAL MODEL FOR INVESTIGATING THE EFFECTS OF THE *FUSARIUM* MYCOTOXIN DEOXYNIVALENOL (DON)

Jamie Marie Hooft  
University of Guelph, 2016  
Advisor: Professor D.P. Bureau

The *Fusarium* mycotoxin deoxynivalenol (DON) is a ubiquitous contaminant of cereal grains. Rainbow trout, a carnivorous species, are highly sensitive to diets containing realistic concentrations of DON (< 1 ppm), whereas omnivorous species (e.g. channel catfish) are able to tolerate much higher levels (e.g. up to 10 ppm) of DON without reduced performance. The basis of the sensitivity of rainbow trout to DON and the efficacy of a commercial feed additive (CFA) were investigated in a series of three experimental trials. In the first trial, rainbow trout fed diets containing purified DON (0-2.1 ppm) or DON from naturally contaminated corn (0-5.9 ppm) experienced decreases in growth performance, carcass composition, and nutrient utilization parameters. The adverse effects of the diets on trout appeared to be exclusively associated with DON and not related to the presence of co-occurring mycotoxins in the grain. Histopathological analysis indicated cellular changes consistent with the ability of DON to activate mitogen-activated protein kinases (MAPKs), notably increases in the number of dead cells and decreases in the number of mitotic cells in the pyloric caeca and/or liver with increasing dietary levels of DON. In a second trial, the inclusion of a CFA in diets containing 0.3 to 2.0 ppm DON was not effective in preventing the negative effects of DON on the
performance of rainbow trout. Finally, a comparative study revealed that Nile tilapia were unaffected by diets containing up to 1.3 ppm DON which otherwise resulted in decreased performance of trout. The species-specific sensitivity to DON could not be explained by differences in the total hepatic UDP-glucuronosyltransferase (UDPGT) activity nor was the response to DON affected by the digestible starch content of the diet (12 or 24%). This finding was in contrast to the hypothesis that the ability of omnivorous species to more effectively utilize dietary carbohydrates compared to carnivorous species may be related to glucuronidation capacity and consequently, sensitivity to DON. This thesis indicates that DON contamination of fish feeds has the potential to result in deleterious clinical and subclinical effects in some species. Furthermore, rainbow trout may be a valuable model species for future investigation.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>15-ADON</td>
<td>15-acetyl DON</td>
</tr>
<tr>
<td>3-ADON</td>
<td>3-acetyl DON</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>aaMF</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>ADC</td>
<td>Apparent digestibility coefficient</td>
</tr>
<tr>
<td>ADG</td>
<td>Average daily gain</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>Aflatoxin B$_1$</td>
<td>AFB$_1$</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AME</td>
<td>Alternariol monomethyl ether</td>
</tr>
<tr>
<td>AOH</td>
<td>Alternariol</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein transcription factor</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
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<tr>
<td>ARG</td>
<td>Arginase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVSA</td>
<td>Apparent villus surface area</td>
</tr>
<tr>
<td>b.w.</td>
<td>Body weight</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT enhancer-binding protein</td>
</tr>
<tr>
<td>caMF</td>
<td>Classically activated macrophages</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CFA</td>
<td>Commercial feed additive</td>
</tr>
<tr>
<td>CGM</td>
<td>Corn gluten meal</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase</td>
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CP  Crude protein
CPC  Corn protein concentrate
CREB  cAMP response element binding protein
CSF  Cerebral spinal fluid
CTA  Conditioned taste aversion
CTZ  Chemoreceptor trigger zone
CYP1A  Cytochrome P450 1A
CYP450  Cytochrome P450
D3G  DON-3-glucoside
DAS  Diacetoxyscirpenol
DCNB  1,2-dichloro-4-nitrobenzene
DM  Dry matter
DNA  Deoxyribonucleic acid
DOM-1  De-epoxy deoxynivalenol
DON  Deoxynivalenol
DON-3-GlcA  DON-3-β-ᴅ-O-glucuronide
DON-7-GlcA  DON-7-β-ᴅ-O-glucuronide
DON-8-GlcA  DON-8-β-ᴅ-glucuronide
dsRNA  Double-stranded RNA
EC  Enterochromaffin cells
EFSA  The European Food Safety Authority
E-GM  Esterified-glucomannan
eIF2  Eukaryotic initiation factor 2
ELEM  Equine leukoencephalomalacia
ELISA  Enzyme-linked immunosorbent assay
EM  Egyptian montmorillonite
ER  Endoplasmic reticulum
ERE  Energy retention efficiency
ERK 1/2  Extracellular signal regulated protein kinases
EROD  Ethoxyresorufin O-deethylase
FA  Fusaric acid
FBW  Final body weight
FCR  Feed conversion ratio
FE  Feed efficiency
FHB  Fusarium head blight
FI  Feed intake
FKRH  Forkhead transcription factor
FSR  Fractional synthesis rate
Fumonisin B₁  FB₁
Fumonisins  FUM
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD</td>
<td>Glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDDY</td>
<td>Grain Distiller’s Dried Yeasts</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GE</td>
<td>Gross energy</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyltransferase</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric inhibitory peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GMA</td>
<td>Glucosmannon adsorbent</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HSI</td>
<td>Hepatosomatic index</td>
</tr>
<tr>
<td>Hck</td>
<td>Hematopoietic cell kinase</td>
</tr>
<tr>
<td>HMP</td>
<td>Hexose monophosphate shunt</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HSCAS</td>
<td>Hydrated sodium calcium aluminosilicate</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IBW</td>
<td>Initial body weight</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK 1/2</td>
<td>c-Jun N-terminal kinases 1 and 2</td>
</tr>
<tr>
<td>K₂EDTA</td>
<td>Ethylenediaminetetraacetic acid dipotassium salt dehydrate</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium phosphate monobasic</td>
</tr>
<tr>
<td>Kₘ (Kₐ)</td>
<td>Substrate concentration at half maximum velocity</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin-4 receptors</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MDE</td>
<td>Mycotoxin degrading enzyme</td>
</tr>
<tr>
<td>MED</td>
<td>Minimum emetic dose</td>
</tr>
</tbody>
</table>
Met-tRNAi
MFP
MIP
miRNA
MON
MROD
n
MFP
Mycofix® Plus
Macrophage inflammatory protein
microRNA
Moniliformin
Methoxyresorufin O-demethylase
Hill coefficient
Nicotinamide adenine dinucleotide
Nicotinamide adenine dinucleotide phosphate
Sodium hydroxide
Nitrogen-free extract
Nuclear factor kappa B
Nivalenol
No-observed-adverse-effect-level
Neuropeptide Y
Neutral red
Nitrogen retention efficiency
Nucleus tractus solitarius
Ochratoxin A
Ochratoxin alpha
Polycyclic aromatic hydrocarbon
Phosphate-buffered saline
Polymerase chain reaction
Prostaglandin E2
Double-stranded RNA associated protein kinase
p-nitrophenol
Pro-opiomelanocortin
Pancreatic polypeptide
Porcine pulmonary edema
Pyrophosphate
Pentose phosphate pathway
Protein retention efficiency
Pentoxyresorufin O-depenthylase
Paraventricular nucleus
Peptide tyrosine tyrosine
Recovered energy
Retained nitrogen
Ribonucleic acid
Ribosomal ribonucleic acid
Ribotoxic stress response
SAPK  Stress-activated protein kinases
SBM  Soybean meal
SGR  Specific growth rate
siRNA  Small interfering RNA
SOD  Superoxide dismutase
SPC  Soy protein concentrate
Stg  Sterigmatocystin
$t_{1/2}$  Elimination half-life
TCA  Trichloroacetic acid
TEER  Trans-epithelial electrical resistance
TGC  Thermal-unit growth coefficient
TNF-α  Tumor necrosis factor alpha
TRP  Tryptophan
UDP  Uridine 5′-diphosphate
UDPGA  Uridine diphosphate glucuronic acid
UDPGT  UDP-glucuronosyltransferase
UGT1A  UDP-glucuronosyltransferase 1A
UTP  Uridine triphosphate
$V_d$  Volume of distribution
VH  Villus height
VLDLs  Very-low-density lipoproteins
Vmax  Maximum reaction rate
VW  Villus width
WBC  White blood cell
Z4G  Zearalenone 4-β-d-glucopyranoside
ZON  Zearalenone
α-MSH  α-melanocyte stimulating hormone
β-NF  β-naphthoflavone
CHAPTER 1 – GENERAL INTRODUCTION

The high cost and volatile supply of fish meal and fish oil are major constraints limiting their use in commercial fish feeds (Tacon and Metian, 2008; Naylor et al., 2009; FAO, 2014). Consequently, more economical high quality plant-derived ingredients including soybean meal (SBM), corn gluten meal (CGM), canola meal, grain and oilseed protein concentrates and a number of grain by-products (e.g. wheat bran, rice bran, wheat shorts, Dried Distillers Grains with Solubles) are increasingly relied upon to formulate feeds for a wide variety of intensively farmed carnivorous finfish species (Hardy, 2010). The successful inclusion of significant levels of plant-based ingredients in aquaculture feeds is largely the result of a continually evolving understanding of nutritional requirements, more accurate assessment of ingredient chemical composition, the effective application of processing techniques to overcome potential limitations of raw materials (e.g. anti-nutritional factors, high fibre content) and the use of feed additives including supplemental nutrients such as amino acids and inorganic phosphorous as well as enzymes, prebiotics/probiotics and organic acids (Bureau, 2004; Glencross et al., 2007; Hardy, 2010; NRC, 2011). Despite these advancements, use of plant protein sources in aquaculture feeds is not without challenges. In particular, the inherent potential for contribution of these ingredients to mycotoxin contamination of fish feeds has received increasing attention over the last decade (Manning, 2005; Spring and Fegan, 2005; Sissener et al., 2011; Sanden et al., 2012). Nonetheless, knowledge regarding the effects of mycotoxins on commercially relevant aquaculture species remains relatively sparse.

Mycotoxins are naturally occurring and potentially highly toxic secondary metabolites produced by filamentous fungi. Mycotoxin contamination resulting from fungal infection of
agricultural crops and commodities is closely related to environmental conditions, particularly temperature and moisture, and may occur at various stages of production (in the field, during harvest or processing and in storage; Whitlow et al., 2010). In general, mycotoxins are chemically and thermally stable, rendering them unsusceptible to routine ingredient and feed manufacturing processes such as thermal treatment (drying, roasting), steam-pelleting and extrusion (Bennett and Richard, 1996). Several factors including changing climate patterns (e.g. increased precipitation or drought), expanded use of no-till farming and increased globalization of commodity trade between regions with different mycotoxin contamination profiles and/or regulatory standards have increased the risk of mycotoxin contamination of animal feeds (Dill-Macky and Jones, 2000; Wu, 2004; Schaafsma and Hooker, 2007). Furthermore, existing mycotoxin concentrations may be increased up to three-fold during the production of ethanol co-products such as dried distiller’s grains with solubles (DDGS), possibly limiting the use of these increasingly important economical feedstuffs (Wu and Munkvold, 2008; Schaafsma et al., 2009). In 2003, the Council for Agricultural Science and Technology (CAST) estimated that 25% of the world’s crop production was contaminated by mycotoxins resulting in annual costs to U.S. agriculture of $932 million, $466 million and $6 million due to crop losses, mitigation efforts and reduced livestock productivity or mortality, respectively (CAST, 2003). More current risk assessment analyses suggest that financial losses associated with mycotoxicoses in animals may be considerably higher (Wu, 2007).

Deoxynivalenol (DON), a *Fusarium* mycotoxicin belonging to a structurally related group of compounds known as the trichothecenes, is extensively recognized as a predominant risk to animal productivity and health mainly due to its high prevalence and widespread occurrence in
many different commodities and regions worldwide (Pestka and Smolinski, 2005; Pestka, 2007). A recent global survey of more than 4,200 samples of a variety of feedstuffs indicated that 59% of samples analyzed contained detectable levels of DON. In 2013, worldwide average concentrations of DON in samples of whole grain corn, wheat, CGM, SBM and DDGS were 0.7, 1.1, 0.9, 0.4 and 1.2 ppm, respectively. Regionally, North America had the highest average concentration of DON in tested samples (1.3 ppm; Nährer and Kovalsky, 2014). Little information on the DON content of finished fish feeds is currently available, but findings of a small-scale survey identified detectable levels of DON in 80% of commercial carp feeds collected in central Europe at concentrations up to 0.8 ppm (Pietsch et al., 2013).

Clinically, the most common signs of chronic feed-borne exposure to DON in experimental animals are reduced feed intake and refusal, reduced weight gain, altered nutritional efficiency and immunotoxicity. Acute physiological effects resulting from consumption of DON-contaminated feeds include vomiting, diarrhea, intestinal inflammation and gastrointestinal hemorrhage. In general, the order of decreasing sensitivity to DON among terrestrial species is: swine > rats > mice > poultry ≈ ruminants (Rotter et al., 1996). Reduced feed intake and weight gain have been observed in growing pigs fed diets containing 1 to 2 ppm DON (Young et al., 1983), whereas wheat contaminated with 5 ppm DON did not affect the performance, productivity or health of broiler chickens, laying hens and turkey poults (Hamilton et al., 1985a,b). Histopathology associated with DON exposure is variable between studies and highly dependent on the route, dose and duration of exposure. Effects ranging from extensive necrosis of the gastrointestinal tract, hepatocytes, lymphoid tissues and bone marrow to focal lesions of kidney and cardiac tissue have been observed in rodents administered high (> 10
mg/kg body weight) intraperitoneal doses of DON (Forsell et al., 1987; Sahu et al., 2008). In contrast, no significant lesions were found in mice and rats or weanling pigs fed diets contaminated with 6 ppm DON for 18 weeks or 2 ppm DON for 4 weeks, respectively (Arnold et al., 1986; Dänicke et al., 2008).

Despite a limited number of studies, differences in response to DON-contaminated feeds have also been noted between fish species. Juvenile channel catfish fed diets containing up to 10 ppm DON from a purified source or naturally contaminated wheat did not experience reductions in feed intake, feed efficiency or growth performance (Manning, 2005). In comparison, rainbow trout appear to be extremely sensitive to feed-borne DON. Highly significant linear decreases in feed intake, weight gain, growth rate (thermal-unit growth coefficient, TGC), feed efficiency and nitrogen and energy utilization efficiencies were observed in rainbow trout fed five diets containing increasing, graded levels of naturally occurring DON ranging from 0.3 to 2.6 ppm. Pair-feeding suggested that decreased performance was related to deleterious metabolic effects and not simply the result of reduced feed intake. Interestingly, the sensitivity of rainbow trout to DON in terms of its effect on weight gain appears to be comparable to or even greater than that of starter pigs (Hooft et al., 2011). Preliminary evaluation of selected tissues indicated morphological changes of the livers of some fish fed the contaminated diets consistent with those observed in other species at much higher levels of DON (Tiemann et al., 2006b; Hooft et al., 2011). Consequently, systematic evaluation of histopathological alterations related to feed-borne exposure to DON in rainbow trout may offer interesting insight into the subclinical mechanisms of toxicity of this ubiquitous contaminant at commercially relevant concentrations. Furthermore, there is a critical need for research
investigating the application of strategies to alleviate the highly significant reductions in growth performance of rainbow trout exposed to feeds naturally contaminated with DON.

Mycotoxin-detoxifying agents are routinely incorporated into feeds to mitigate the effects of mycotoxins on animal health and performance. Feed additives can be broadly categorized into adsorbing agents and biotransforming agents. Mycotoxin adsorbents or binders such as yeast cell wall extracts reduce the bioavailability and subsequently the uptake and systemic distribution of mycotoxins to target organs, while biotransforming agents (e.g. bacteria, fungi and enzymes) are aimed at the degradation of mycotoxins (the parent compounds) into non- or less-toxic metabolites (Boudergue et al., 2009). The efficacy of many different commercially available feed additives in preventing negative growth and health related effects of mycotoxins has been the subject of numerous studies in several species, often with contradictory results (Swamy et al., 2002a,b, 2003; Leung et al., 2007; Girish and Smith, 2008). Feed additives may offer a potential solution to the effects of mycotoxins, particularly DON, on highly sensitive farmed fish species. However, to date, few studies regarding the effectiveness of mycotoxin adsorbents or biotransforming agents in rainbow trout have been conducted.

The basis of the apparent high variability in sensitivity of different fish species (e.g. rainbow trout vs. channel catfish) to DON is largely unknown. Rapid and efficient absorption and a limited capacity to transform DON to de-epoxy DON (DOM-1) in pigs compared to poultry and ruminants via intestinal or rumen microbial activity are widely believed to be critical factors influencing the effects of DON on these species (He et al., 1992; Dänicke et al., 2004a,b). Guan et al. (2009) screened the digesta of more than 60 fish representing nine species and found de-
epoxidation of DON by microorganisms in only one sample obtained from a catfish species after a 72 hour incubation, suggesting that de-epoxidation may not significantly contribute to DON metabolism in fish. Concurrently, increasing evidence has indicated that glucuronidation of DON is extensively involved in its metabolism in humans, animals and plants (Poppenberger et al., 2003; Turner et al., 2011; Warth et al., 2012a, 2013). Hepatic glucuronidation has been proposed to be an important metabolic determinant of interspecies variability in sensitivity to DON (Maul et al., 2012). Therefore, it is hypothesized that species-specific differences in glucuronidation capacity may be related to the substantial differences in sensitivity to DON in fish. The ability of different fish species to effectively utilize dietary carbohydrates may also influence the relationship between glucose metabolism and the endogenous supply of UDP-glucuronic acid as a cofactor for glucuronidation (Bánhegyi et al., 1988; Wilson, 1994; Braun et al., 1997).

1.1 – Objectives

The main objectives of this thesis were: (1) to determine if reduced growth performance of rainbow trout fed diets naturally contaminated with feed-borne Fusarium mycotoxins is primarily associated with DON or rather is the result of synergistic or additive interactions related to the presence of multiple mycotoxins; (2) to characterize the effects of DON on histopathological changes of organs and tissues in rainbow trout; (3) to evaluate the efficacy of a commercial feed additive with adsorbing and biotransforming properties in preventing the adverse effects of DON on rainbow trout; and (4) to compare the effects of DON
on growth and detoxification mechanisms in rainbow trout and Nile tilapia fed diets containing different levels of digestible carbohydrates as a potential nutritional mitigation strategy.
CHAPTER 2 – LITERATURE REVIEW

2.1 – Introduction

Aquaculture is currently one of the fastest growing food producing sectors in the world (Hernández et al., 2012; FAO, 2014). Nonetheless, aquaculture operations and fish feed manufacturers are faced with considerable challenges: stagnant or decreasing marketable product prices, increasing production costs and limited supply and high cost of historically important feed ingredients (i.e. fish meal and fish oil) due to increasing demand for compounded feed to support the rapid growth of the aquaculture sector (Tacon and Metian, 2008; Naylor et al., 2009; Bureau, 2010). Consequently, feed manufacturers must increasingly rely on a variety of more economical and readily available plant-derived protein sources of differing quality (e.g. grain and oilseed milling by-products) in order to better satisfy market demand and improve the cost-effectiveness of their feeds (Hardy, 2010). However, increased inclusion of plant-based ingredients has inadvertently increased the potential for mycotoxin contamination of aquaculture feeds (Manning, 2005; Spring and Fegan, 2005; Sissener et al., 2011; Sanden et al., 2012).

Mycotoxins are naturally occurring secondary metabolites produced primarily by the mycelial structure of filamentous fungi, which are characterized by their potential to elicit undesirable effects in humans and animals following consumption of contaminated foods or feedstuffs (Hussein and Brasel, 2001; Bryden, 2007). General, unspecific symptoms ranging from reduced production efficiency to mortality associated with consumption of mycotoxin-contaminated feeds make diagnosis difficult. Synergistic interactions resulting from the presence of multiple mycotoxins in feedstuffs or opportunistic disease resulting from
compromised immune status may also complicate identification of mycotoxicoses in production animals. Furthermore, sensitivity to mycotoxins varies greatly between species and is dependent on several factors which can modify the expression of toxicity including age, gender and nutritional and health status prior to exposure (Rotter et al., 1996; Whitlow and Hagler, 2002). It is estimated that 25% of the world’s crop production is contaminated with mycotoxins (CAST, 2003). The economic impact of mycotoxins is effectively impossible to quantify; however, risk assessment analyses have indicated that financial losses to U.S. agriculture associated with crop losses, mitigation efforts and reduced livestock productivity or mortality range from $630 million to $2.5 billion per annum (CAST, 2003).

Mycotoxins constitute a structurally and chemically diverse group of more than 300 compounds which vary greatly in their potential toxicity (Bennett and Klich, 2003). The primary classes of mycotoxins with relevance to human health and animal agriculture are produced by fungal species belonging to the genera Aspergillus, Penicillium, and Fusarium. These include aflatoxins, ochratoxins, fumonisins, zearalenone (ZON) and the trichothecenes (CAST, 2003; Marquardt, 1996). Deoxynivalenol (DON), a trichothecene mycotoxin produced by Fusaria species, is of particular concern in animal feed and production industries due to its widespread distribution in a number of geographical regions, high prevalence in cereal grains (mainly corn, wheat and barley) and resistance to thermal processing. In 2013, a large-scale survey of more than 4,200 commodity samples intended for animal feed from over 50 countries worldwide indicated detectable concentrations of DON in 59% of analyzed samples; notably, 42% of all surveyed samples were contaminated with DON at levels above 0.3 ppm (Nährer and Kovalsky, 2014).
Consumption of feedstuffs contaminated with DON results in a variety of adverse effects in experimental and farm animals. Prolonged feed-borne exposure to low doses of DON is commonly associated with anorexia (reduced feed intake and growth), decreased productivity and altered nutritional efficiency. Comparatively, acute exposure to increasing concentrations of DON is related to symptoms including leucocytosis, gastrointestinal hemorrhage, diarrhea and emesis or vomiting (particularly in pigs). Generally, the order of decreasing sensitivity to DON among the most routinely studied species is: swine > rats > mice > poultry ≈ ruminants (Rotter et al., 1996). Reduced feed intake and weight gain have been observed in growing pigs fed diets containing 1 to 2 ppm DON (Young et al., 1983), whereas short-term feeding of a ration containing 66 mg/kg DON did not affect feed intake or milk production in lactating dairy cows (Côté et al., 1986). Differences in the absorption, distribution, metabolism and elimination of DON are widely cited as critical determinants of species-specific sensitivity to DON in terrestrial animals (Pestka, 2007). However, the exact mechanism(s) associated with species differences in sensitivity to DON remain a relatively controversial issue.

Despite limited information, marked species differences in sensitivity to DON have also been observed in fish. Juvenile channel catfish fed diets containing up to 10 ppm DON from a purified source or naturally contaminated wheat did not experience reductions in feed intake, feed efficiency or growth performance (Manning, 2005). In contrast, feed intake, feed conversion ratio (FCR) and growth rate of Atlantic salmon were adversely affected by a diet containing 3.7 ppm DON (Döll et al., 2010). Rainbow trout appear to be extremely sensitive to DON. Highly significant linear or quadratic decreases in feed intake, weight gain, growth rate and feed efficiency were observed in rainbow trout fed diets naturally contaminated with
graded levels of DON ranging from 0.3 to 2.6 ppm (Hooft et al., 2011). The basis of this high sensitivity could not be determined; however, pair-feeding suggested that the adverse effects of DON on the performance of rainbow trout are related to deleterious metabolic disturbances and not strictly due to reduced feed intake. This finding was supported by preliminary (i.e. unsystematic) histopathological examination indicating multifocal areas of fatty infiltration and phenotypically altered hepatocytes in selected livers from fish fed the most highly contaminated diet (2.6 ppm DON; Hooft et al., 2011).

Scientific evidence demonstrating negative effects of DON on the growth performance of high-value commercial aquaculture species at levels routinely detected in raw materials destined for use in feeds has become an issue of increasing concern in fish nutrition. Therefore, the aims of this review are to summarize and discuss the occurrence, toxicity and mechanisms of action of DON and its histopathological effects and metabolism in routinely studied experimental and farm animals. The effects of DON on fish will be presented according to the available information.

2.2 – Natural production and incidence of DON

DON (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) was first isolated as “Rd-toxin” in Japanese barley infected with *Fusarium* fungal species (Morooka et al., 1972) and later by Vesonder et al. (1973) as “vomitoxin” from *Fusarium* infected corn in Northwestern Ohio due to its emetic effect on swine. DON is primarily produced by strains of *Fusarium graminearum* and the closely related species *F. culmorum*, *F. pseduograminearum* and *F. crookwellense* (Desjardins et al., 1993; Glenn, 2007). *F. graminearum* and *F. culmorum*, in
particular, are important etiological agents of DON and plant pathogens frequently associated with Fusarium Head Blight (FHB; also called scab) in small cereal grains (mainly wheat, barley, oats and rye) and Gibberella ear rot (or pink ear rot) in corn produced in temperate regions (Miller and Richardson, 2013). In wheat and corn, a positive correlation between the level of DON and degree of grain infection with *F. graminearum* has been established (Reid et al., 1996). The incidence and severity of *Fusarium* diseases and DON contamination is arguably most influenced by environmental conditions permissive to fungal infection, particularly when appropriate temperatures and above average rainfall or high humidity (moisture) coincide at the time of anthesis (flowering) for wheat and silk emergence for corn (Miller and Richardson, 2013). The timing of precipitation, rather than the amount, appears to be critical to the development of FHB or ear rot. The optimal temperatures for growth of *F. culmorum* and *F. graminearum* are 21 and 26-28 °C, respectively (Reid et al., 1999; Canady et al., 2001). Accordingly, *F. culmorum* is generally found in areas with relatively cool weather during the growing season (e.g. Northern Europe), while *F. graminearum* is predominantly associated with warmer climates including grain-growing regions of North America, China and Japan (Canady et al., 2001). DON is frequently found to naturally co-occur in cereals with mycotoxins produced by these and other *Fusarium* species. A summary of toxigenic *Fusarium* species and their mycotoxins is presented in Table 2.1. Notably, Streit et al. (2012) reported common co-occurrence of DON with its acetylated derivatives, 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON), T-2 toxin, HT-2 toxin, fumonisins, moniliformin and the estrogenic mycotoxin, ZON.

Agronomic variables related to crop management also impact DON contamination of cereal grains. Significant reductions in the incidence of FHB and DON content of wheat were
achieved by plowing crop residues under the soil surface in order to minimize the source of inoculum (Dill-Macky and Jones, 2000). However, evidence suggests that the effect of residual soil surface residue on the development of FHB and DON content of subsequent crops may be more related to the specific substrate (i.e. previous crop) than the amount of residue per se. Using linear models, Schaafsma et al. (2001) attributed 14-28% of the variation in DON content of wheat to the crop grown one season previous depending on year. For example, DON concentrations in wheat crops grown in fields previously containing corn were over two-fold higher on average compared to the levels of DON in wheat grown in rotation with soybeans (Schaafsma et al., 2001). Similarly, Schaafsma and Hooker (2007) reported that cultivar accounted for 25 and 27% of the variation in DON contamination of Ontario-produced corn and wheat, respectively. Use of Bt-corn hybrids, for example, which confer resistance to the European corn borer (Ostrinia nubilalis), significantly reduced the concentration of DON by 59% when extensive stalk injury resulting from a high intensity of insect infestation was observed (Schaafsma et al., 2002).

Numerous studies have consistently reported a high prevalence of DON in commodities used as feed ingredients. From 1979 to 1995, the incidence of DON in Ontario wheat ranged from 22 to 100% (with the exception of one year), during which time the annual mean of positive samples was as high as 0.75 ppm. The same multi-year monitoring program determined DON to be a persistent problem in Ontario corn with yearly incidences of 13 to 100% and annual averages of positives samples up to 1.4 ppm (Scott, 1997). In fact, widespread epidemics of FHB occurred approximately once every nine years from 1927 to 1992 (Schaafsma et al., 2001). Between 2004 and 2011, analysis of more than 17,000 samples of
feeds and feed ingredients collected worldwide indicated that 56% of samples were contaminated with an average of 1 ppm DON (Streit et al., 2013; Schatzmayr and Streit, 2013). This finding was supported by a more recent global survey of more than 4,200 samples of a variety of feedstuffs conducted in 2013. Nearly 60% of samples analyzed contained detectable levels of DON. Worldwide average concentrations of DON in samples of whole grain corn, wheat, CGM, SBM and DDGS were 0.7, 1.1, 0.9, 0.4 and 1.2 ppm, respectively. Regionally, Northern Europe and North America had the highest average concentration of DON in tested samples (1.5 and 1.3 ppm, respectively; Nährer and Kovalsky, 2014). Currently, limited information regarding the contamination of finished aquaculture feeds with DON is available. However, a small-scale survey identified detectable levels of DON in 80% of commercial carp feeds collected in central Europe at concentrations up to 0.8 ppm (Pietsch et al., 2013).
Table 2.1. Mycotoxigenic *Fusarium* species, their frequency of occurrence, phytopathogenicity and associated mycotoxins (adapted from Chelkowski, 1998; Eriksen, 2003; Glenn, 2007; Miller and Richardson, 2013).

<table>
<thead>
<tr>
<th><em>Fusarium</em> species</th>
<th>Occurrence</th>
<th>Pathogenicity</th>
<th>Mycotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. graminearum</em></td>
<td>+++</td>
<td>+++</td>
<td>DON, ADON, ZON, NIV, FUS-X,</td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>+++</td>
<td>+++</td>
<td>DON, ZON, NIV, FUS-X, FUS-C, ADON</td>
</tr>
<tr>
<td><em>F. crookwellense</em></td>
<td>+</td>
<td>+++</td>
<td>ZON, DON, NIV, FUS-X, FUS-C</td>
</tr>
<tr>
<td><em>F. sporotrichioides</em></td>
<td>+</td>
<td>++</td>
<td>T-2, HT-2, DAS, NEO, FUS-C</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>++</td>
<td>++</td>
<td>DAS, MAS, NIV, FUS-X, T-2, HT-2, FUS-C</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>+++</td>
<td>++</td>
<td>MON, BEA, FP</td>
</tr>
<tr>
<td><em>F. avenaceum</em></td>
<td>+++</td>
<td>++</td>
<td>MON, FUS-C</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>+</td>
<td>+</td>
<td>FUM, MON, BEA, FP</td>
</tr>
<tr>
<td><em>F. moniliforme</em></td>
<td>+++</td>
<td>++</td>
<td>FUM, FUS-C</td>
</tr>
<tr>
<td><em>F. tritripectum</em></td>
<td>+</td>
<td>+</td>
<td>MON, FUS-C</td>
</tr>
<tr>
<td><em>F. acuminatum</em></td>
<td>+</td>
<td>+</td>
<td>T-2, HT-2, MON, DAS, MAS, NEO</td>
</tr>
</tbody>
</table>

*a* Synonymous with *F. cerealis*

*b* Synonymous with *F. verticillioides*

*c,d* +++ high; ++, moderate; +, low

*e* Main mycotoxins produced. Abbreviations: ADON, 15-acetyl-deoxynivalenol and/or 3-acetyl-deoxynivalenol; BEA, Beauvericin; DAS, diacetoxyscirpenol; DON, deoxynivalenol; FP, fusaproliferin; FUM, fumonisins; FUS-C, fusarin C; FUS X, fusarenon X; HT-2, HT-2 toxin; MAS, monoacetoxysoicirpenol; MON, moniliformin; NEO, neosolaniol; NIV, nivalenol; T-2, T-2 toxin; ZON, zearalenone.
2.3 – Chemical structure and properties of trichothecenes

The trichothecenes are a group of approximately 180 closely related sesquiterpenoids with a characteristic tricyclic nucleus (trichothecene) and varying degrees of cytotoxic potency (Figure 2.1; Table 2.2). All trichothecene mycotoxins contain an epoxide group at the C-12,13 position and most also have a C-9,10 double bond which are the characteristics responsible, in large part, for their toxicological activity (Sudakin, 2003; Pestka and Smolinski, 2005). Trichothecenes are generally classified into four categories (types A-D) according to their functional groups, as well as the fungal species which produce them. Type A (e.g. T-2 toxin, HT-2 toxin, diacetoxyxirpenol) and type B (e.g. DON, nivalenol) trichothecenes are distinguished by the absence or presence of a carbonyl group at the C-8 position, respectively. Both type A and type B trichothecenes are produced by fungal species belonging to the genera Fusarium. In contrast, type C and type D trichothecenes are formed by other taxonomically unrelated genera of fungi (e.g. Myrothecium, Stachybotrys) and can be complex macrocyclic esters. Type C trichothecenes (e.g. crotocin, baccharin) contain an additional epoxide group at the C-7,8 or C-9,10 position, while type D trichothecenes (e.g. satratoxin, roridin) have a macrocyclic ring between C-4 and C-15 with two ester linkages. Epidemiological surveys indicate that that relative to the widespread distribution of type A and type B trichothecenes in cereal grains, type C and type D trichothecenes rarely occur in food and feed (Ueno, 1984; Krška et al., 2001; Sudakin, 2003).

Trichothecenes are non-volatile, low molecular weight (250-550 g/mol) compounds generally resistant to degradation by light and temperature. In particular, DON is stable at 120 °C, moderately stable at 180 °C and partially stable at 210 °C. In general, complete thermal
inactivation of trichothecenes requires heating at approximately 480 °C for 10 minutes. These compounds are unaffected at neutral and acidic pH and the C-12,13 epoxide group is resistant to nucleophilic attack. Most trichothecenes are soluble in solvents including acetone, chloroform and ethylacetate. DON and other highly hydroxylated trichothecenes are also soluble in more polar solvents such as acetonitrile, methanol, ethanol and water. The thermal and chemical stability of trichothecenes make them a particular concern in food/feed processing industries (Hazel and Patel, 2004).
Table 2.2. Substituent groups ($R_1$-$R_5$) of selected type A and type B trichothecene mycotoxins corresponding to the chemical structure depicted in Figure 2.1 (Ueno et al., 1973; Ohta et al., 1978; Ehrlich and Daigle, 1987).

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$R^3$</th>
<th>$R^4$</th>
<th>$R^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-3</td>
<td>C-4</td>
<td>C-15</td>
<td>C-7</td>
<td>C-8</td>
</tr>
<tr>
<td>Type A:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>OCOCH$_2$CH(CH$_3$)$_2$</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>H</td>
<td>OCOCH$_2$CH(CH$_3$)$_2$</td>
</tr>
<tr>
<td>Diacetoxyscirpentriol (DAS)</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Neosolaniol (NEO)</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Scirpentinol</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Monoacetoxyscirpentriol (MAS)</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Type B:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>=O</td>
</tr>
<tr>
<td>3-acetyl-DON (3-ADON)</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>=O</td>
</tr>
<tr>
<td>15-acetyl-DON (15-ADON)</td>
<td>OH</td>
<td>H</td>
<td>OAc</td>
<td>OH</td>
<td>=O</td>
</tr>
<tr>
<td>Nivalenol (NIV)</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>=O</td>
</tr>
<tr>
<td>Fusarenon X</td>
<td>OH</td>
<td>OAc</td>
<td>OH</td>
<td>OH</td>
<td>=O</td>
</tr>
</tbody>
</table>

=, double bond
OAc, acetoxy group
2.4 – Molecular and cellular mechanisms of action

2.4.1 – Inhibition of protein synthesis

The toxicity of trichothecenes is partially explained by the disruption of eukaryotic protein synthesis, particularly in highly proliferating cells and tissues (Kiessling, 1986; Pestka, 2010a). Inhibitory activity requires the presence of an intact C-9,10 double bond and integrity of the C-12,13 epoxide group (Wei and McLaughlin, 1974). Reduction of the epoxide ring (de-epoxidation) results in the apparent loss of toxicity (Feinberg and McLaughlin, 1989). Trichothecenes are able to bind to the ribosome and interfere with the action of peptidyl transferase. Binding at the peptidyl transferase site appears to be affected by the size of the nascent polypeptide chain in the P or A site (peptidyl-tRNA and aminoacyl-tRNA, respectively). Highly substituted trichothecenes (e.g. T-2 toxin) can bind only at peptidyl transferase centers in which the nascent peptide is less than three to four amino acids. In contrast, smaller trichothecenes (e.g. DON) are able to bind to most or all peptidyl transferase centers (Ehrlich and Daigle, 1987). The degree of substitution is further related to the traditional classification of trichothecenes according to their ability to inhibit the different steps of translation: initiation (I-type), elongation (E-type) or termination (T-type) (Carter and Cannon, 1977; Cundliffe and Davies, 1977). Structure-activity studies have indicated that trichothecenes with substituents at both C-3 and C-4 predominantly inhibit polypeptide chain initiation (e.g. T-2 toxin, DAS), whereas those lacking one substituent at either site (e.g. DON) generally inhibit elongation and/or termination (Ehrlich and Daigle, 1987).

More recent studies have uncovered additional mechanisms potentially involved in the impairment of protein synthesis by DON. Using a murine macrophage cell line, Zhou et al.
found that DON rapidly activates double-stranded RNA-associated protein kinase (PKR) resulting in phosphorylation of eukaryotic initiation factor 2α (eIF2α). Phosphorylation of eIF2 on its α subunit, in turn, is known to convert eIF2-GDP into a competitive inhibitor of the 5-subunit guanine nucleotide exchange factor (GEF), eIF2B. Consequently, formation of the ternary complex between eIF2, GTP and initiator methionyl transfer RNA (Met-tRNA) is decreased and initiation is inhibited (Sonenberg and Hinnebusch, 2009). A study using the same cell line indicated that DON can promote intracellular cleavage of 28S rRNA by binding to specific sites within the peptidyl transferase center and facilitating the action of constitutive or inducible RNases (Li and Pestka, 2008). Damage to rRNA could subsequently affect ribosomal function and translation. In addition, DON is able to upregulate a large number of microRNAs (miRNAs) (He and Pestka, 2010). Several studies have demonstrated that miRNAs either inhibit translation and/or destabilize the mRNA (Sonenberg and Hinnebusch, 2009).

The inhibition of protein synthesis by DON has been confirmed in vivo in different species. For example, intraperitoneal (i.p.) injection of rather high doses of purified DON (10, 20 and 80 mg/kg live weight) in mice inhibited incorporation of labelled leucine in the heart. Doses of 20 and 80 mg/kg live weight further inhibited protein synthesis in the kidney, liver and spleen (Robbana-Barnat et al., 1987). Chowdhury and Smith (2004) observed a significant reduction in hepatic protein synthesis of laying hens fed a diet containing 11.9 ppm DON and 1.1 ppm 15-ADON. Likewise, employment of the flooding dose technique using [2H5]-phenylalanine revealed a significant reduction in protein synthesis expressed as fractional synthesis rate (FSR) in the kidneys, spleen and ileum of pigs fed a diet containing 5.7 ppm DON for 4 weeks. However, FSR of several other organs and tissues in pigs including the liver,
skeletal muscle, heart and pancreas was not affected by chronic or acute exposure to DON (Dänicke et al., 2006).

2.4.2 – Ribotoxic stress response

It has been well-established that DON and other translational inhibitors can rapidly activate mitogen-activated protein kinases (MAPKs) in a process known as the ribotoxic stress response (RSR; Iordanov et al., 1997; Laskin et al., 2002). MAPKs are a large family of proline-directed serine/threonine kinases involved in important cell signalling pathways which mediate physiological responses including gene expression, cell proliferation, differentiation, survival, motility and apoptosis. Conventional MAPKs are comprised of three sub-families: the extracellular signal regulated kinases 1 and 2 (ERK 1/2), p54 and p46 c-Jun N-terminal kinases 1 and 2 or stress-activated protein kinases (JNK 1/2 or SAPK) and p38 MAPK isoforms (Fang and Richardson, 2005; Cargnello and Roux, 2011).

Chemical inhibitor and gene silencing studies have revealed that hematopoietic cell kinase (Hck) and double-stranded RNA-(dsRNA)-activated protein kinase (PKR) play critical roles in the RSR and are important upstream mediators of DON-induced MAPK phosphorylation. PKR is a widely expressed serine/threonine protein kinase that can be activated by dsRNA, interferon, cytokines and oxidative stress and functions as a signal integrator for pathways leading to JNK and p38 activation. PKR was initially identified to inhibit translation via phosphorylation of eIF2α as part of an antiviral response. Hck is a member of the Src family of cytoplasmic protein kinases which transduces extracellular signals that regulate critical cellular processes such as proliferation, differentiation, migration and cytokine upregulation (Pestka,
DON rapidly activated PKR and Hck within 1 to 5 min in a murine macrophage cell line (Zhou et al., 2003a, 2005a). Pretreatment of macrophages with inhibitors of PKR or use of a human monocyte cell line containing an antisense PKR expression vector markedly impaired MAPK phosphorylation following the rank order JNK > p38 > ERK (Zhou et al., 2003a). Similarly, use of Src family tyrosine kinase inhibitors suppressed the DON-induced phosphorylation of Hck and concentration-dependently impaired phosphorylation of ERK, JNK and p38 and selected downstream substrates. Inhibition or knockdown of Hck with siRNAs also reduced DON-induced tumor necrosis factor alpha (TNF-α) production and caspase-3 activation (Zhou et al., 2005a).

Two possible mechanisms for the initiation of the RSR by DON have been proposed to date. First, DON-mediated damage of the peptidyl transferase region of the 28S rRNA may initiate activation of kinases and their recruitment to the ribosome. Direct interaction of PKR, Hck and p38 with the 40S ribosomal subunit was demonstrated by immunoblot analysis following sucrose density gradient fractionation in human monocyte or murine macrophage cell cultures. In this sense, the ribosome may act as a scaffold in the DON-induced RSR (Bae and Pestka, 2008; Bae et al., 2010). Secondly, the interaction of DON with the ribosome may elicit an endoplasmic reticulum (ER) stress response. Degradation of GRP78, a well-known ER chaperone which stabilizes unfolded proteins, in DON-treated macrophages was attenuated by preincubation with inhibitors of cathepsins and calpains suggesting that DON initiates autophagy of unfolded proteins targeted by GRP78. Furthermore, DON was found to increase ATF6 and IRE1α, ER stressor sensors released by GRP78 upon accumulation of unfolded proteins in the ER lumen. Some evidence suggests that ATF6 and IRE1α may be involved in
mediating MAPK phosphorylation and upregulating proinflammatory gene expression (Shi et al., 2009).

2.4.3 – Proinflammatory response

Depending on dose and duration of exposure or exposure frequency, DON can induce immunostimulation or immunosuppression by upregulating proinflammatory gene expression or apoptosis, respectively. Acute, low dose exposure aberrantly affects immune function by initiating a rapid and transient upregulation of cytokines, chemokines and other proinflammatory-related proteins. DON induced mRNA expression of TNF-α, interleukin (IL)-6, macrophage inflammatory protein (MIP)-2 and cyclooxygenase 2 (COX-2) in macrophages (Moon and Pestka, 2002; Chung et al., 2003a,b; Jia et al., 2004), IL-8 in monocytes (Gray and Pestka, 2007; Gray et al., 2008) and IL-2 in T cells (Li et al., 1997). Likewise, induction of various cytokines and chemokines was demonstrated in vivo using a mouse model (Kinser et al., 2004).

Upregulation of mRNA expression of proinflammatory genes by DON involves transcriptional and post-transcriptional processes, importantly transcription factor activation and mRNA stabilization. For example, expression of components of the activator protein (AP)-1 transcription factor complex (c-Fos, Fra-2, c-Jun and Jun B) were significantly increased in splenic tissue following oral dosing of mice with 25 mg DON/kg body weight (Kinser et al., 2004). Similarly, DON increased binding activity of the transcription factors AP-1, nuclear factor kappa B (NF-κB) and CCAAT enhancer-binding protein (C/EBP) in vitro (Ouyang et al., 1996; Li et al., 2000; Wong et al., 2002; Gray and Pestka, 2007). AP-1, NF-κB and C/EBP transcription factors have been extensively implicated in the regulation of cytokine gene expression (Pestka,
2010b). Alternatively, DON has also been shown to enhance the mRNA stability of TNF-α (Chung et al., 2003b), IL-6 (Jia et al., 2006) and COX-2 (Moon et al., 2003) in a macrophage cell line, IL-8 in human epithelial and intestinal cells (Choi et al., 2009) and IL-2 in murine EL-4 thymoma cells (Li et al., 1997). Enhanced mRNA stability relates to the presence of AUUUA pentamers in the 3’-untranslated region (UTR) of mRNA, which target transcripts for rapid degradation. Involvement of the AU-rich element (ARE) in DON-induced mRNA stability has been demonstrated for COX-2 and IL-8 (Moon et al., 2003; Choi et al., 2009; Pestka, 2010b).

MAPK activation appears to be a necessary precedent for transcription factor activation or mRNA stabilization and enhanced cytokine production. Using inhibitors specific to ERK and p38, Moon and Pestka (2002) demonstrated suppression of DON-induced prostaglandin E₂ (PGE₂) and COX-2 protein expression in murine macrophages. Similarly, inhibition of p38 and ERK blocked or significantly reduced the DON-induced activity of a luciferase reporter gene driven by the murine TNF-α promoter (Chung et al., 2003b). In agreement, (Zhou et al., 2003b) were able to temporally relate MAPK phosphorylation, transcription factor activation and cytokine mRNA expression in vivo using a mouse model. Maximal phosphorylation of JNK 1/2 and p38 were observed in spleen cells 15 to 30 min following oral gavage of mice with 25 mg DON/kg body weight, whereas ERK 1/2 activation was more prolonged with detectable phosphorylation in the spleen and thymus from 15 to 120 min post-gavage. These findings are consistent with the kinetics of labelled DON in the mouse which reached maximal levels in spleen, plasma and other tissues within 30 min following oral administration (Azcona-Olivera et al., 1995). Increased binding activity of one AP-1 complex and C/EBP was brief and occurred concurrently with or immediately subsequent to phosphorylation of the MAPKs (30 min post-
gavage). Conversely, maximal activation of other transcription factors including a second AP-1 complex, cAMP response element binding protein (CREB) and NF-kB was later and more prolonged (4 to 8 hours post-gavage). The mRNA expression of the proinflammatory cytokines of interest (TNF-α, IL-1β and IL-6) were significantly elevated at 3 and 6 hours post-treatment and returned to control levels after 9 hours. The authors proposed that the prolonged ERK response relative to JNK and p38 may have been receptor-driven via the production of cytokines, suggesting a potential external signalling pathway (Zhou et al., 2003b). Taken together, these findings appear to be consistent with the capacity of DON to induce a proinflammatory response via sequential events: (1) phosphorylation of MAPKs (2) transcription factor activation and (3) cytokine expression.

2.4.4 – DON-induced cell death and survival

In contrast to the aforementioned stimulatory effects, high concentrations of DON can promote rapid onset of apoptosis leading to immunosuppression. Apoptosis of the thymus, bone marrow, spleen and Peyer’s patches was observed in mice given an oral gavage of 25 mg DON/kg body weight (Zhou et al., 2000). However, a lesser dose (12.5 mg/kg body weight) did not significantly affect the extent of thymic apoptosis, suggesting that high concentrations of DON are required to induce cell death of lymphoid tissue in the mouse (Islam and Pestka, 2006). Induction of apoptosis was also reported in murine lymphoid cultures of T, B and IgA+ cells incubated with 50 μg DON/mL for 8 hours (Pestka et al., 1994). Comparatively, in cultured macrophages a significant increase in caspase-3 activity indicative of apoptosis was observed after incubation with 250 ng DON/mL for 3 hours (Yang et al., 2000; Zhou et al., 2003a, 2005a).
Caspase-3 activity was similarly elevated in primary cultures of porcine hepatocytes treated with 1, 10 or 100 μg DON/mL in a dose-dependent manner (Mikami et al., 2004). In support of this finding, more than 100 μmol DON/L (29.6 μg DON/mL) was required to induce apoptosis in a human intestinal cell line (Maresca et al., 2002).

Despite this variability in the apoptotic response of different cell lines and animal models, the involvement of MAPKs in the intrinsic mitochondrial pathway of DON-induced apoptosis has been convincingly demonstrated. DON-exposed macrophages exhibited increased caspase 3-dependent DNA fragmentation which was suppressed by p38 inhibition. Downstream of p38, p53 served as an important linkage in the DON-induced intrinsic apoptotic pathway (Zhou et al., 2005b). The regulatory role of this pathway in DON-induced apoptosis has also been confirmed in human colon carcinoma cells (Bensassi et al., 2009). In response to death stimuli, BAX and Bad (pro-apoptotic proteins) translocate to the mitochondrial membrane and form a pro-apoptotic complex with Bcl-2 or Bcl-xL (anti-apoptotic proteins), resulting in the formation of pores in the mitochondrial membrane followed by cytochrome C release (Downward, 2004; Zhou et al., 2005b). Indeed, Western blot analysis of cytoplasmic and mitochondrial cellular fractions indicated increased BAX and decreased cytochrome C in the mitochondrial fraction of DON-treated cells. Concurrent with apoptosis, DON was found to activate two survival pathways. In contrast to p38, ERK inhibition potentiated caspase 3-dependent DNA fragmentation by DON. DON-induced phosphorylation of p90RSK and its downstream substrate, Bad, were demonstrated to be ERK-dependent. Additionally, DON upregulated Akt and two of its substrates, glycogen synthase kinase-3β (GSK-3β) and forkhead transcription factor (FKRH) (Zhou et al., 2005b). Phosphorylation of Bad can decrease its
interaction with anti-apoptotic members of the Bcl-2 family and thereby inhibit the release of cytochrome C. Similarly, phosphorylation of GSK-3β and FKHR contribute to cell survival by promoting storage of glucose as glycogen (through a poorly understood mechanism) and suppressing transcription of pro-apoptotic genes, respectively (Downward, 2004; Zhou et al., 2005b). Accordingly, the extent of apoptosis resulting from DON exposure is proposed to be determined by the balance of these competing apoptotic and survival pathways (Zhou et al., 2005b; Pestka, 2010b).

2.4.5 – Cell cycle arrest

In addition to cell death, the cytotoxicity of DON is also related to its interference with normal cell cycle progression. Fornelli et al. (2004) observed reduced viability of an insect cell line related to DON-induced cell cycle arrest in the G₀/G₁ phase. In accordance with this finding, treatment of porcine uterine cells with DON resulted in G₀/G₁ phase arrest and reduced the entry of cells into S phase (Tiemann et al., 2003). The cyclin-dependent kinase inhibitor p21, a downstream substrate of p53, has been linked to alteration of cell cycle progress in response to DON. Human epithelial cells exposed to DON underwent dose-dependent G₂/M phase arrest associated with elevated gene expression of p21. Total p53 protein level was not affected by treatment with DON in epithelial cells, suggesting p53-independent p21 induction. Relative to the confirmed increased in p21 transcriptional activity, upregulation of ERK 1/2 resulting in p21 mRNA stabilization was determined to be an important contributing factor to DON-induced cell cycle disruption (Yang et al., 2008).
The effects of DON on cell proliferation have been demonstrated in vivo. Feeding a diet containing 2.8 ppm DON for 35 days significantly reduced the number of mitotic figures in the enterocytes of 5-week-old piglets (Bracarense et al., 2012). A significant reduction in the average number of mitotic figures in the intestinal crypts of the jejunum and ileum was also observed in piglets of the same age following consumption of a diet containing 1.5 ppm DON for 4 weeks. Interestingly, feeding diets containing NIV (1.3 ppm) and ZON (1.5 ppm) in addition to higher concentrations of DON (2 or 3 ppm) did not significantly affect the number of mitotic figures in the intestinal crypts relative to the control group (Gerez et al., 2015). Grenier et al. (2011) reported increased development of megalocytosis or gross enlargement of the hepatocytes in weaned piglets fed a diet contaminated with 2.8 ppm DON isolated from *F. graminearum*. Megalocytes have been described as morphologically and functionally viable cells in the process of hypertrophy. That is, these cells are able to grow, but not divide, a phenomenon consistent with cell cycle arrest and the anti-mitotic effects of DON (Svoboda et al., 1971; Zimmerman, 1999). Conversely, increased numbers of mitotic figures proposed to be associated with an adaptive increase in intestinal absorptive surface area were present in the duodenal and jejunal crypts of young broiler breeders fed a diet containing up to 3.8 ppm DON (Girgis et al., 2010). These findings suggest that the anti-proliferative effects of DON differ between species and may be affected by the presence and/or concentrations of other mycotoxins in the diet.
2.4.6 – Other molecular and cellular effects

Trichothecenes have been shown to inhibit DNA and RNA synthesis. For example, DNA synthesis was inhibited by approximately 70% in Caco-2 cells incubated with 10 μM DON for 24 hours (Kouadio et al., 2005, 2007). The 50% inhibitory concentration for DNA synthesis in this cell line was 1.7 μM. Interestingly, at concentrations above 5 μM, inhibition of DNA synthesis appeared to be reversed such that an inhibition of only 45% was observed at a concentration of 20 μM DON (Kouadio et al., 2005). The exact mechanism for the inhibition of DNA synthesis is not fully understood. Some researchers suggest that it is a secondary effect resulting from protein synthesis inhibition or apoptosis; conversely, others have proposed that impairment of nucleic acid synthesis occurs independently of these processes (Brunner and Morris, 1988; Thompson and Wannemacher Jr., 1990; Kouadio et al., 2005).

In addition to ribosome-mediated effects, trichothecenes can impair cell membrane function. Brunner and Morris (1988) attributed adverse effects of low doses of T-2 toxin on myoblast cell membranes including suppressed glucose transport, impaired calcium and rubidium uptake and reduced intracellular lactate dehydrogenase (LDH) activity to an affinity of T-2 toxin for the phospholipid bilayer and secondary changes in membrane transport. Still, several studies have indicated that cellular damage is often potentiated by oxidative stress: an imbalance favouring pro-oxidants and/or disfavouring anti-oxidants (Chandra et al., 2000). Indeed, increased lipid peroxidation and altered antioxidant enzyme activities have been observed in different cell lines and animal models exposed to DON or T-2 toxin (Suneja et al., 1989; Kouadio et al., 2005, 2007; Hou et al., 2013). Rizzo et al. (1994) reported decreased hepatic activities of superoxide dismutase (SOD) and catalase (CAT) and reduced hepatic
glutathione (GSH) content in rats administered a single oral dose of DON or T-2 toxin (28 or 3.6 mg/kg b.w., respectively) which could be effectively counteracted by dietary selenium, α-tocopherol and ascorbic acid. Sustained elevation of superoxide anion (O$_2^-$) and dose- and time-dependent changes in SOD, CAT, glutathione peroxidase (GPx), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PD) activities of human embryonic kidney (Hek-293) cells incubated with 2.5 or 5 μM DON were later described (Dinu et al., 2011). SOD is an important first-line defence against free radicals, enabling the dismutation of O$_2^-$ to hydrogen peroxide (H$_2$O$_2$), which can then be converted to water and oxygen by CAT and GPx. GR, in turn, restores the availability of GSH by catalyzing the reduction of glutathione disulfide (GSSH) to GSH using NADPH generated primarily by G6PD as part of the pentose phosphate pathway (PPP; Suneja et al., 1989; Dinu et al., 2011).

The potential contribution of oxidative stress to apoptosis has been extensively reviewed (Chandra et al., 2000). Whether or not induction of oxidative stress associated with exposure to DON influences its apoptotic capacity appears to be a point of contention. Lutein, a natural carotenoid and powerful antioxidant, was effective in preventing DON-induced nuclear translocation of NF-κB in HT-29 cells. NF-κB is cited as an important regulator of cell proliferation and programmed cell death in addition to its role in the proinflammatory response. Furthermore, lutein prevented changes in cellular morphology characteristic of apoptosis including membrane blebbing and chromatin condensation which were otherwise observed in DON-treated cells (Krishnaswamy et al., 2010). However, Bensassi et al. (2009) reported p53 and caspase-dependent apoptosis in the same cell line despite negligible increases in the production of reactive oxygen species (ROS).
Figure 2.2. Molecular and cellular effects of DON depicting pathways leading to the proinflammatory response (green), apoptosis (orange) and cell cycle arrest (purple). Cell survival pathway indicated with dashed line. Block arrow across bottom represents outcomes associated with increasing dose and duration of exposure. R=ribosome; PI=proinflammatory.
2.5 – Effects of DON on terrestrial species

2.5.1 – Acute toxicity

Acute toxicity studies are usually aimed at targeting a specific toxicological outcome in order to provide insight into a potential mechanism of action or valuable information for hazard assessment (Pestka and Smolinski, 2005). High dose, short-term exposure of experimental animals to DON is generally associated with signs including abdominal distress (e.g. intestinal inflammation, gastrointestinal hemorrhage), increased salivation, malaise, diarrhea and emesis (Forsyth et al., 1977; Young et al., 1983; Pestka, 2010a). Acute toxicity experiments have been particularly useful in evaluating the potent emetic capacity of DON. The minimum emetic dose (MED) for DON in pigs was determined to be 50 μg/kg body weight (b.w.) following intraperitoneal (i.p.) injection with a no-observed-adverse-effect-level (NOAEL) of 25 μg/kg b.w.

Orally, the MED and NOAEL for DON in pigs ranged from 50-100 μg/kg b.w. and 25-75 μg/kg b.w., respectively (Forsyth et al., 1977; Forsell et al., 1987). Conversely, a much higher MED of 10 mg DON/kg b.w. (administered subcutaneously) was observed in 10-day-old ducklings indicating significant variation in terms of species-specific response to DON (Yoshizawa and Morooka, 1974).

Despite lack of a vomiting reflex, mice and rats have been widely used to inform risk assessment and regulatory guidelines. LD$_{50}$ (median lethal dose) values have been routinely used as an experimental endpoint within this context. Acute exposure to extremely high doses of DON is often associated with shock-like death in rodents (Pestka, 2010a). Mouse LD$_{50}$ values (mg/kg b.w.) for DON were estimated to be 78 by oral gavage and 43-49 b.w. (i.p.). Relative to DON, its acetylated precursors, 3-ADON and 15-ADON, appear to have similar toxicities in both
mice and pigs (Thompson and Wannemacher Jr., 1986; Forsell et al., 1987; Pestka et al., 1987). Comparatively, LD$_{50}$ values (mg/kg b.w.) for T-2 toxin and nivalenol in adult male mice ranged from 2.1-10.5 and 4.1-6.3, respectively, depending on the route of exposure (Ueno, 1984). Nonetheless, the emetic and anorectic effects of DON are equivalent to or exceed those described for more acutely toxic trichothecenes (Rotter et al., 1996). Furthermore, based on its consistent and widespread prevalence in agricultural commodities, chronic, low-dose exposure to DON represents a substantial concern in animal production industries.

2.5.2 – Effects on growth and performance parameters

The most prominent clinical effects of prolonged dietary exposure of animals to DON at more practically relevant (i.e. naturally occurring) concentrations are decreased weight gain, reduced feed intake (i.e. anorexia) and altered nutritional efficiency (Pestka and Smolinski, 2005). The effect of DON on growth and other performance indices varies greatly between species. Generally, the order of decreasing sensitivity to DON among routinely studied farm and experimental animals is considered to be: swine > rats > mice > poultry ≈ ruminants (Rotter et al., 1996).

In growing pigs, decreases in feed intake and live weight gain have been observed at 1 to 2 ppm DON, whereas levels of 12 and 20 ppm led to nearly complete feed refusal and vomiting, respectively (Forsyth et al., 1977; Young et al., 1983; Abbas et al., 1986). Significant linear and quadratic reductions in average daily gain (ADG), feed intake and feed efficiency were observed in piglets with an average weight of 7 kg fed diets containing 0.1, 1.3, 2.6, 5.1, 6.4, 7.8, 8.6 and 11.9 ppm DON for 3 weeks. Notably, the diet contaminated with 1.3 ppm DON
markedly decreased ADG and feed consumption in these animals (Young et al., 1983). This result is in contrast to that of Chavez (1984) who did not report any significant reductions in weight gain or feed intake of weanling pigs with the same initial weight fed diets containing up to 1.7 ppm DON from naturally contaminated wheat. Differences in the mycotoxin profiles of naturally contaminated ingredients have been implicated as an important contributing factor to such discrepancies between studies. Likewise, no adverse effects on the performance of larger pigs (25 kg) were associated with diets containing 0.5 and 1 ppm DON from naturally contaminated oats during an 8 week experimental period. However, a dose-related decrease in weight gain was evident in animals fed diets contaminated with approximately 2 and 4 ppm DON, while feed intake and feed efficiency were also negatively affected in those groups fed the diet containing 4 ppm DON (Bergsjø et al., 1992). Despite these adverse effects on weight gain, feed intake and feed efficiency, no significant impact of diets containing 2.9 and 5.3 ppm DON on nitrogen digestibility or nitrogen retention efficiency (NRE) were observed in barrows (Friend et al., 1986a).

In contrast to pigs, poultry and ruminants appear to be highly tolerant to DON. Incorporation of contaminated winter or spring wheat into diets for laying hens at levels up to 5 ppm DON had no effect on feed intake, feed efficiency, egg production and yield, egg quality or hatchability (Hamilton et al., 1985a). No negative effects of diets naturally contaminated with 5 ppm DON on performance parameters and absolute or relative organ weights were observed in young Leghorn chickens (between 7 and 35 days of age) or broiler chicks (Hamilton et al., 1985b; Awad et al., 2006a). Indeed, Awad et al. (2004) demonstrated that broiler chickens were able to tolerate starter and grower diets containing 10 ppm purified DON over six weeks
without any adverse impacts on body weight, feed consumption or feed conversion ratio (FCR) despite impaired intestinal glucose transport. Several studies have also demonstrated a transient stimulatory effect of DON-contaminated feeds containing as much as 10 ppm DON on weight gain and feed intake of laying hens and broiler chickens (Hamilton et al., 1985b; Swamy et al., 2002a; Awad et al., 2004). Like poultry, cattle appear to be highly tolerant to DON. For example, feeding a diet containing 66 ppm DON for five days did not affect the body weight, feed consumption, milk production or milk composition of lactating dairy cows (Côté et al., 1986). Similarly, ad libitum intake of hay supplemented with a wheat-oat concentrate containing 6.4 ppm DON for six weeks did not have any deleterious impact on body weight of non-lactating Holsteins, despite slightly lower feed consumption compared to a concentrate contaminated with 1.5 ppm DON (Trenholm et al., 1985).

2.5.3 – Histopathological and morphological effects

Histopathological and morphological effects of DON are variable between species and may differ significantly according to the route, dose and duration of exposure. Outcomes of histological evaluation, in particular, are subject to the assessment criteria employed (Gibson-Corley et al., 2013; Klopfleisch, 2013). For example, use of an incidence approach (absolute frequency of occurrence) as opposed to ordinal scoring (severity factor × extent) may have contributed to different conclusions regarding lesions associated with DON-contaminated feeds in piglets despite similar experimental conditions (Dänicke et al., 2008; Grenier et al., 2011). In the same sense, specialized stains (e.g. periodic acid-Schiff or Berlin blue) and/or microscopy techniques (e.g. electron microscopy) can provide greater or more specific insight into
mechanisms of organ and tissue pathology than routine methods (Bergsjø et al., 1993; Goyarts et al., 2005; Tiemann et al., 2006a,b; Tiemann et al., 2008a,b). It is important to carefully consider these factors when interpreting the available literature. Table 2.3 provides a summary of selected studies concerning the histopathological and morphological effects of DON in terrestrial species.

Rodent models have been widely used to characterize pathologies associated with high dose exposure to DON. Acute oral gavage or i.p. administration of high concentrations of DON (60-100 mg/kg b.w.) resulted in necrosis of intestinal epithelial cells, bone marrow, thymus, spleen and heart in B6C3F1 weanling female mice (Forsell et al., 1987). Arnold et al. (1986) verified the development of lymphatic and gastrointestinal lesions in mice orally gavaged with 2.5 or 7.5 mg DON/kg b.w. for 5 weeks. Cardiac lesions were also observed in 4 to 6 week old BALB/c mice fed diets containing 10 and 20 ppm DON equating to 1.5 and 3 mg/kg b.w./d, respectively (Robbana-Barnat et al., 1987). In contrast, evaluation of the brain, lungs, thymus, esophagus, stomach, duodenum, jejunum, ileum, kidneys, liver, pancreas, spleen, mesenteric lymph nodes and gonads in male Swiss-Webster mice or Sprague-Dawley rats fed diets containing 6.3 ppm DON for 18 weeks did not reveal any distinct pathologies (Arnold et al., 1986). Similarly, Iverson et al. (1995) did not report any meaningful lesions in B6C3F1 mice fed diets containing up to 10 ppm DON in a two-year investigation.

Unlike rodents, evidence of organ and tissue dysfunction has been noted in pigs exposed to concentrations of DON not exceeding 10 ppm. Prepuberal gilts and pregnant sows fed diets contaminated with 3.1 to 9.6 ppm DON and low levels of ZON (up to 0.4 ppm) for 5 weeks experienced decreased liver glycogen content and hemosiderosis of the liver and spleen:
increased accumulation of hemosiderin without marked tissue damage (Tiemann et al., 2006a,b; Tiemann et al., 2008a,b). The authors proposed that hemosiderosis, in the absence of anemia or decreased erythrocyte counts, may have resulted from sequestration of damaged erythrocytes in splenic sinusoids or increased erythrocyte turnover and impaired iron utilization related to overexpression of certain cytokines induced by DON (Dänicke et al., 2005; Tiemann et al., 2006a,b; Tiemann et al., 2008a,b). Ultrastructural examination of liver tissue in gilts and sows fed diets containing 6.1 or 9.6 ppm DON revealed proliferation of the smooth ER, loss of bound ribosomes from the rough ER and an increase in fatty vacuoles (Tiemann et al., 2006b, 2008a). Grenier et al. (2011) confirmed the appearance of cytoplasmic vacuoles in the livers of piglets fed a diet contaminated with 2.8 ppm purified DON, although the nature of the vacuolation was not elaborated.

Damage to the rough ER and fatty change (steatosis) of the liver are important criteria of exposure to certain drugs and hepatotoxic agents. Disposal of lipids by the liver involves oxidation of fatty acids in the mitochondria and movement of triglycerides out of the liver. The latter requires coupling of the triglycerides with an apolipoprotein and phospholipids to form very-low-density lipoproteins (VLDLs) that serve to transport lipids from the liver to the tissues (Zimmerman, 1999). Given that the ultrastructure of the mitochondria was unaffected in sows and gilts fed up to 9.6 ppm DON, defective synthesis of apolipoprotein resulting from degranulation of the rough ER, rather than impaired mitochondrial oxidation, appears to be responsible for fatty infiltration (vacuolation) of liver tissue (Tiemann et al., 2006b, 2008a; Zimmerman, 1999). Like the rough ER, the smooth ER is especially vulnerable to hepatotoxic agents. Hypertrophy of the smooth ER is described as an adaptive response to hepatic injury.
usually accompanied by an initial increase in metabolic capacity; however, prolonged exposure to some inducing agents may ultimately lead to dissociation between the quantity of smooth ER and its function, with decreased activities of enzymes involved in detoxification reactions despite increased amounts of smooth ER (Zimmerman et al., 1999).

In contrast to the extensive intestinal necrosis associated with high dose exposure to DON in rodents, the effects of more practically relevant levels of DON on the gastrointestinal tract of swine appear to be limited to altered morphology of the stomach. Weaned barrows fed diets naturally contaminated with 3 or 4 ppm DON had increased corrugation of the fundic region of the stomach compared to control ad libitum and control pair-fed pigs (Rotter et al., 1994, 1995). Similarly, increased thickness and a higher degree of folding, apparently indicative of a “healthier” mucosa, were reported in the esophageal area of the stomach of pigs fed diets contaminated with up to 6 ppm DON (Friend et al., 1986b; Rotter et al., 1992; Rotter et al., 1994). Rotter et al. (1996) attributed the effects of DON on the stomach mucosa of pigs to its ability to delay gastric emptying (Fioramonti et al., 1993). On the other hand, changes in morphometric indices of the duodenum and jejunum of broiler chicks, particularly decreased villus height (VH) and villus width (VW) in birds fed diets containing 5 to 10 ppm DON, are suggestive of gastrointestinal irritation (Awad et al., 2006a,b). Girish and Smith (2008) reported comparable changes in duodenal and jejunal, but not ileal, morphometric parameters of turkeys fed diets containing 2.2 or 3.3 ppm DON from naturally contaminated ingredients during the starter and grower phases, respectively. Using similar diets, researchers from the same group later described an increase in VH and apparent villus surface area (AVSA) in the jejunum and ileum of female broiler breeders which they suggested represented a
compensatory displacement of nutrient absorption to the distal intestine. Increased crypt depth and hyperplasia of the crypt epithelium in DON-fed birds may also reflect an adaptive need for increased absorptive surface area (Girgis et al., 2010). The compensatory morphological changes of the distal intestine are in agreement with the ability of poultry to sustain high concentrations of DON without depressed performance (Girish and Smith, 2008; Girgis et al., 2010).
Table 2.3. Histopathological and morphological effects of DON on terrestrial species and related *in vitro* models.

<table>
<thead>
<tr>
<th>DON level(s)</th>
<th>DON source</th>
<th>Species or <em>in vitro</em> model</th>
<th>Route of exposure</th>
<th>Duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3 mg/kg diet</td>
<td>Contaminated wheat</td>
<td>Mice, Swiss-Webster</td>
<td>Oral (feed)</td>
<td>18 weeks</td>
<td>No significant pathological lesions</td>
<td>Arnold et al. (1986)</td>
</tr>
<tr>
<td>6.3 mg/kg diet</td>
<td>Contaminated wheat</td>
<td>Rats, Sprague-Dawley</td>
<td>Oral (feed)</td>
<td>18 weeks</td>
<td>No significant pathological lesions</td>
<td>Arnold et al. (1986)</td>
</tr>
<tr>
<td>0.75, 2.5 or 7.5 mg/kg b.w./day</td>
<td>Purified in solvent</td>
<td>Mice, Swiss-Webster</td>
<td>Gavage</td>
<td>5 weeks</td>
<td>Significant mortality/morbidity at 2.5 and 7.5 mg/kg; Dose-dependent ↓ in thymocytes and cortical thickness of thymus and congestion of the splenic red pulp with reduced haematopoietic cells; 7.5 mg/kg: spleens small with few or no lymphatic nodules present, narrowing of the periarteriolar lymphocytic sheath of the spleen, necrosis of the mesenteric lymph nodes, Peyer’s patches small with ↓ lymphocytes and germinal centers, necrosis of enterocytes and ↓ height and number of villi in the jejunum</td>
<td>Arnold et al. (1986)</td>
</tr>
<tr>
<td>5.0 mg/kg diet</td>
<td>NC wheat</td>
<td>Broiler chicks</td>
<td>Oral (feed)</td>
<td>3 weeks</td>
<td>No histological lesions of the crop, gizzard, jejunum, caecum and ileum; ↓ VH and VW in the duodenum, but VH and VW not affected in jejunum</td>
<td>Awad et al. (2006a)</td>
</tr>
<tr>
<td>10 mg/kg diet</td>
<td>Purified</td>
<td>Broiler chicks</td>
<td>Oral (feed)</td>
<td>6 weeks</td>
<td>No gross pathological lesions and no abnormalities of the crop, proventriculus, ileum, cecum or rectum; Slight villus atrophy and irregular crypts in some birds; ↓ VH and VW in the duodenum and jejunum</td>
<td>Awad et al. (2006b)</td>
</tr>
<tr>
<td>0, 0.7, 1.7 or 3.5 mg/kg diet</td>
<td>NC oats</td>
<td>Pigs, Norwegian Landrace</td>
<td>Oral (feed)</td>
<td>13 weeks</td>
<td>Minor foci of purulent inflammatory lesions in the terminal ileal Peyer’s patches and mesenteric lymph nodes independent of dietary treatment</td>
<td>Bergsjø et al. (1993)</td>
</tr>
<tr>
<td>2.3 mg/kg diet</td>
<td>Contaminated triticale</td>
<td>Piglets (weaned)</td>
<td>Oral (feed)</td>
<td>4 weeks</td>
<td>No significant differences in the absolute frequencies of histopathological lesions of the liver, spleen, kidneys, pancreas or heart</td>
<td>Dänicke et al. (2008)</td>
</tr>
</tbody>
</table>
### Table 2.3 continued. Histopathological and morphological effects of DON on terrestrial species and related *in vitro* models.

<table>
<thead>
<tr>
<th>DON level(s)</th>
<th>DON source</th>
<th>Species or <em>in vitro</em> model</th>
<th>Route of exposure</th>
<th>Duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/kg b.w. (gavage) or 60 mg/kg b.w. (i.p.)</td>
<td>Purified produced from <em>F. graminearum</em></td>
<td>Mice, B6C3F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Single oral gavage or i.p. injection</td>
<td>2 weeks</td>
<td>Necrosis of intestinal crypt epithelial cells, edema and congestion of the lamina propria of the villi; Lymphoid necrosis in the spleen and thymus, ↓ lymphocytes around the periarterial lymphatic sheath of the spleen, ↑ nuclear debris and pyknotic nuclei in the red and white pulp of the spleen, ↓ density of lymphocytes and epithelial cells of the medulla of the thymus, karyolysis of lymphocyte nuclei; Necrosis of the haematopoietic and myelopoietic elements of the bone marrow; Focal to locally extensive areas of myocardial cell necrosis</td>
<td>Forsell et al. (1987)</td>
</tr>
<tr>
<td>2.2 (starter), 3.3 (grower), 3.1 (developer) or 2.9 (finisher) mg/kg diet</td>
<td>NC corn and wheat</td>
<td>Turkey poult, hybrid</td>
<td>Oral (feed)</td>
<td>12 weeks</td>
<td>Starter: ↓ VH in duodenum and jejunum, ↓ AVSA in jejunum; Grower: ↓ VW in duodenum, ↓ VH in jejunum, ↓ AVSA in duodenum and jejunum, ↓ submucosa thickness of ileum; Developer and finisher: no significant effects</td>
<td>Girish and Smith (2008)</td>
</tr>
<tr>
<td>2.5 (starter) or 3.8 (finisher) mg/kg diet</td>
<td>NC corn and wheat</td>
<td>Broiler breeder female chicks, Ross 308</td>
<td>Oral (feed)</td>
<td>10 weeks</td>
<td>VH: duodenum ↓, jejunum ↑, ileum ↑; CD: duodenum ↑, jejunum ↑; C: V: duodenum ↑, jejunum ↑, ileum ↓; AVSA: duodenum ↓, jejunum ↑, ileum ↑; ↑ intraepithelial lymphocytes in duodenum, ↑ CD associated with cryptal hyperplasia and mitotic figures in the duodenum and jejunum</td>
<td>Girgis et al. (2010)</td>
</tr>
<tr>
<td>6.2 (grower) or 6.8 (finisher) mg/kg diet</td>
<td>Contaminated wheat</td>
<td>Pigs, German Landrace x Pietrain</td>
<td>Oral (feed)</td>
<td>11 weeks</td>
<td>Non-relevant macroscopic lesions in the kidneys independent of dietary treatments; No macroscopic lesions of the liver or spleen</td>
<td>Goyarts et al. (2005)</td>
</tr>
</tbody>
</table>
Table 2.3 continued. Histopathological and morphological effects of DON on terrestrial species and related *in vitro* models.

<table>
<thead>
<tr>
<th>DON level(s)</th>
<th>DON source</th>
<th>Species or <em>in vitro</em> model</th>
<th>Route of exposure</th>
<th>Duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8 mg/kg diet</td>
<td>Purified</td>
<td>Piglets (weaned)</td>
<td>Oral (feed)</td>
<td>5 weeks</td>
<td>↑ lesion score in liver and kidney; Liver: disorganization of hepatic cords, cytoplasmic and nuclear vacuolization and megalocytosis; Kidney: cytoplasmic and nuclear vacuolization of tubular epithelial cells and lymphocyte infiltration</td>
<td>Grenier et al. (2011)</td>
</tr>
<tr>
<td>0.2, 1, 5, 10 and 30 μM</td>
<td>Purified</td>
<td>Pig jejunal explant culture</td>
<td>Incubation</td>
<td>4 hours</td>
<td>10 and 30 μM: autolysis of tissue; 0.2-5 μM: dose-dependent ↑ of pyknotic nuclei in enterocytes, flattening and coalescent villi, ↓ VH and edema and necrosis in the lamina propria; 5 μM: apoptosis, diffuse sloughing of epithelial lining from villi surface and coating of secreted mucus and cellular debris along the explant surface</td>
<td>Kolf-Clauw et al. (2009)</td>
</tr>
<tr>
<td>100 μM</td>
<td>Purified</td>
<td>Human intestinal cells</td>
<td>Incubation</td>
<td>48 hours</td>
<td>Cellular apoptosis</td>
<td>Maresca et al. (2002)</td>
</tr>
<tr>
<td>0.01, 0.1, 1, 10 or 100 μg/mL</td>
<td>Purified</td>
<td>Piglet hepatocyte culture</td>
<td>Incubation</td>
<td>24 hours</td>
<td>Marked cell death in 1, 10 and 100 μg/mL groups; Dead hepatocytes were shrunken and round, detached from the culture plate and had chromatin-condensed and/or fragmented nuclei; &gt;80% of detached hepatocytes were stained by TUNEL indicating DNA fragmentation of the nuclei</td>
<td>Mikami et al. (2004)</td>
</tr>
<tr>
<td>0, 0.75, 1.5, 3.0 mg/kg diet</td>
<td>NC corn</td>
<td>Weaned barrows, Yorkshire</td>
<td>Oral (feed)</td>
<td>4 weeks</td>
<td>Linear ↑ in mucosa thickness of esophageal area; Trend toward linear ↑ in esophageal folding; ↑ fundic corrugation in pigs fed 3 ppm DON vs. pair-fed controls;</td>
<td>Rotter et al. (1994)</td>
</tr>
<tr>
<td>4.0 mg/kg diet</td>
<td>NC corn</td>
<td>Weaned barrows, Yorkshire</td>
<td>Oral (feed)</td>
<td>6 weeks</td>
<td>↑ degree of mucosal corrugation in the fundic region of the stomach of pigs fed the DON diet compared to controls and pair-fed controls</td>
<td>Rotter et al. (1995)</td>
</tr>
<tr>
<td>10 mg/kg b.w.</td>
<td>Purified</td>
<td>Rats, Sprague-Dawley</td>
<td>Single i.p. injection</td>
<td>3, 24 and 72 hours</td>
<td>3 h: disruption in the uniform radiation of sinusoids from the central vein, degenerative changes (homogeneity of cytoplasm) and enlargement of hepatocytes lining the sinuses and necrosis of several hepatocytes; 72 h: liver similar to control</td>
<td>Sahu et al. (2008)</td>
</tr>
</tbody>
</table>
Table 2.3 continued. Histopathological and morphological effects of DON on terrestrial species and related *in vitro* models.

<table>
<thead>
<tr>
<th>DON level(s)</th>
<th>DON source</th>
<th>Species or <em>in vitro</em> model</th>
<th>Route of exposure</th>
<th>Duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 (control), 3.1, 6.1, 9.6 mg/kg diet</td>
<td><em>Fusarium</em> contaminated wheat</td>
<td>Prepubertal gilts, Landrace</td>
<td>Oral (feed)</td>
<td>5 weeks</td>
<td>6.1 and 9.6 mg/kg: ↑ Fe2+ particles (deposition of hemosiderin) in splenocytes vs. controls; No abnormalities in ultrastructure of splenocytes</td>
<td>Tiemann et al. (2006a)</td>
</tr>
<tr>
<td>0.2 (control), 3.1, 6.1, 9.6 mg/kg diet</td>
<td><em>Fusarium</em> contaminated wheat</td>
<td>Prepuberal gilts, Landrace</td>
<td>Oral (feed)</td>
<td>5 weeks</td>
<td>Dose-dependent: ↓ in hepatic glycogen; ↑ in Fe2+ particles in hepatocytes of pigs fed diets containing 3.1, 6.1 and 9.6 mg/kg DON vs. controls; 6.1 and 9.6 mg/kg: ↑ thickness of interlobular connective tissue of the liver with increased collagen fibrils, ↑ autophagosomes, residual bodies and fatty droplets in the hepatic cytoplasm, ↓ bound ribosomes from the ER of the hepatocytes (↑ smooth cisterns); 9.6 mg/kg: fluid-filled vesicles originating from ER; No signs of cellular injury in mitochondria of hepatocytes</td>
<td>Tiemann et al. (2006b)</td>
</tr>
<tr>
<td>9.6 mg/kg diet</td>
<td><em>Fusarium</em> contaminated wheat</td>
<td>Sows (pregnant), Landrace</td>
<td>Oral (feed)</td>
<td>5 weeks (day 75 to 110 of gestation)</td>
<td>Piglets: no significant effects on hepatocytes or splenocytes; Sows: ↑ Fe2+ particles in hepatocytes and red pulp of spleen, autophagosomes, residual bodies and fatty droplets in the cytoplasm of hepatocytes and loss of bound ribosomes from hepatocyte ERs (smooth cisternae); No change in mitochondria of hepatocytes or in ultrastructure of splenocytes</td>
<td>Tiemann et al. (2008a)</td>
</tr>
<tr>
<td>4.4 mg/kg diet</td>
<td><em>Fusarium</em> contaminated triticale</td>
<td>Sows (pregnant), German Landrace</td>
<td>Oral (feed)</td>
<td>5 weeks (day 35 to 70 of gestation)</td>
<td>Piglets: ↑ hepatic glycogen and atrophy of some hepatocytes marked by cytoplasm with swollen mitochondria in which rupture or loss of cristae were often found; Sows: ↑ Fe2+ particles in the red pulp of the spleen</td>
<td>Tiemann et al. (2008b)</td>
</tr>
</tbody>
</table>

NC, naturally contaminated; VH, villi height; VW, villi width; AVSA, apparent villus surface area; CD, crypt depth; C:V, crypt:villus ratio; ER, endoplasmic reticulum
2.6 – Effects of DON on fish

2.6.1 – Effects on growth and performance parameters

Research efforts examining the effects of DON on commercially important fish species are limited relative to other farmed animals. Nevertheless, continual optimization of diet formulation often favouring increased inclusion of plant ingredients has increased awareness regarding the potential risk of inadvertently exposing farmed fish to mycotoxins. Accordingly, a small, but growing, number of studies have focused on providing insight into the effects of DON on growth performance of fish (Table 2.4).

In the earliest published study on this topic, Woodward et al. (1983) reported that diets containing graded levels of DON ranging from 1.0 to 12.9 ppm from artificially infected corn (also noted to contain 4 ppm ZON and a trace of 7-deoxyvomitoxin) caused significant reductions in live weight gain (12 to 92% of the control) and feed intake and increased feed conversion ratio (FCR) of juvenile rainbow trout (with an initial average weight of 50 g/fish). Rainbow trout appeared to be affected by low, practically relevant levels (< 3 ppm) of DON; however, the response of fish at these concentrations was quite highly variable and difficult to accurately determine.

More recently, Hooft et al. (2011) convincingly demonstrated highly significant linear or quadratic decreases in feed intake, weight gain, growth rate (expressed as thermal-unit growth coefficient, TGC), feed efficiency, energy retention efficiency (ERE) and nitrogen retention efficiency (NRE) of rainbow trout with an initial body weight (IBW) of 24 g/fish fed diets containing increasing, graded levels of DON (0.3, 0.8, 1.4, 2.0 and 2.6 ppm) from naturally contaminated corn for 8 weeks. Interestingly, fish pair-fed the control diet (0.3 ppm DON) had
significantly higher TGC, FE and whole body crude protein (CP) content compared to their counterparts fed the diet containing 2.6 ppm DON, indicating that the effects of DON on the performance of rainbow trout are not simply due to reduced feed intake, but rather, related to deleterious metabolic effects. Furthermore, no significant differences were observed in the apparent digestibility coefficients (ADC) of CP or gross energy of smaller fish (IBW=8.5 g/fish) fed diets contaminated with 0.3 to 2.0 ppm DON. These authors indicated that the effect of DON on weight gain is potentially greater in rainbow trout compared to starter pigs when DON intake is considered on a metabolic body weight basis. Similarly, the effect of DON on the performance of salmonid species has also been demonstrated in Atlantic salmon. Feeding fish (IBW=405 ± 31 g/fish) a diet containing 3.7 ppm DON resulted in a significant decrease in feed intake (20%) and specific growth rate (SGR, 31%) and an increase in FCR (18%) compared to fish fed a control diet containing 0.3 ppm DON. In contrast, diets containing increasing, lower concentrations of ZON (0.06 to 0.77 ppm) or OTA (0.09 to 0.33 ppm) did not affect feed intake or growth of Atlantic salmon (Döll et al., 2010).

In fish, like other animals, considerable variability in sensitivity to DON has been observed between species. Generally, warm water omnivorous species are considered more tolerant to DON-contaminated feeds than cold water carnivorous species (Spring and Fegan, 2005; Spring and Burel, 2008; Manning and Abbas, 2012). Most notably, channel catfish (*Ictalurus punctatus*) appear to be much more resistant to DON than salmonids. Similar findings were obtained in an additional trial conducted using diets formulated with naturally-contaminated wheat. In the same study, only fish fed diets containing 15 or 17.5 ppm DON experienced significant reductions in weight gain and significantly poorer FCR compared to the
control group (Manning, 2005). Pietsch et al. (2014a,b) did not observe any negative effect of diets containing less than 1 ppm purified DON on weight gain of common carp (*Cyprinus carpio*). Conversely, significant linear decreases in weight gain, TGC, feed intake and FE of juvenile red tilapia (*Oreochromis niloticus × O. mossambicus*) were associated with diets containing 0.07 to 1.15 ppm DON from naturally contaminated wheat. However, these diets were co-contaminated with several other relatively unknown *Fusarium* metabolites including aurofusarin, culmorin and 15-hydroxyculmorin at concentrations similar to or greater than DON (Tola et al., 2015). The potential contribution of these mycotoxins to the depressed growth performance observed could therefore not be excluded. Nonetheless, broad species categorization with regard to DON sensitivity (i.e. cold water vs. warm water) may be somewhat arbitrary. Continued research is necessary to delineate the basis of species-specific sensitivity to DON in finfish in order to develop accurate regulatory guidelines and industry recommendations.

### 2.6.2 – Histopathological and morphological effects

To date, few in depth evaluations of the histopathological effects of DON in fish have been conducted. The majority of work in this area has been narrowly focused on the liver as the target organ of DON toxicity. Moreover, to our knowledge, just three studies (Pietsch et al., 2014b, 2015; Tola et al., 2015) have reported their results based on a semi-quantitative approach; others provide only qualitative descriptions of histological change (Hooft et al., 2011; Matejova et al., 2014) and/or use biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) as surrogate
indicators of organ and tissue damage (summarized in Table 2.4). Consequently, there is a critical need for studies employing systematic approaches to characterizing histopathological changes in multiple tissues and organs of fish exposed to feed-borne DON. Such work may be particularly useful in better understanding the significant reductions in growth performance of highly sensitive species (e.g. rainbow trout).

Table 2.5 presents an overview of histological changes in fish exposed to DON-contaminated diets. Gross examination of juvenile rainbow trout (IBW=24 g/fish) revealed subcapsular hemorrhage of the liver in some fish fed naturally contaminated diets containing 1.4, 2 or 2.6 ppm DON for 8 weeks (Hooft et al., 2011). The same macroscopic lesion was evident in one-year-old rainbow trout (IBW≈180 g/fish) after short-term feeding of an experimental diet contaminated with 2 ppm purified DON without any substantial histopathological effects (Matejova et al., 2014). Conversely, in the former study, Hooft et al. (2011) described histological changes of the liver including subcapsular edema, fatty infiltration and phenotypically altered hepatocytes in a number of DON-exposed fish. Cytoplasmic vacuolation, subcapsular edema and areas of focal necrosis were similarly reported in the livers of some red tilapia (IBW=4.3 g/fish) fed diets containing 0.3 to 1.2 ppm from naturally contaminated wheat (Tola et al., 2015). Despite these observations, the authors did not find any significant correlation between increasing levels of DON and histopathological changes using a standardized assessment protocol developed by Bernet et al. (1999). Following a 4-week period, Pietsch et al. (2014b) identified increased severity of hyperaemia in the livers of common carp fed diets containing 0.35, 0.62 and 0.95 ppm DON compared to control-fed fish. Fat aggregation and dilation of sinusoids were significantly greater in certain DON-fed groups;
conversely, glycogen content, the extent of vacuolation and the severity of (unspecified) lesions were not affected. A 2-week recovery phase during which time fish were fed the uncontaminated control diet did not appear to be sufficient to reverse the histopathological effects (Pietsch et al., 2014b). However, in a subsequent study, the severity of similar alterations in liver histology was significantly increased in carp fed the diet contaminated with 0.95 ppm compared to their control-fed counterparts after 14 and 26 days, but not after 56 days. This suggests the potential for adaptation or tissue repair following detrimental effects of feed-borne exposure of fish to DON (Pietsch and Burkhardt-Holm, 2015).

Liver injury in carp receiving DON-contaminated feeds has been proposed to be a consequence of lipid peroxidation measured as increased malondialdehyde (MDA) production (Pietsch et al. 2014b; Pietsch and Burkhardt-Holm, 2015). Although free radical-mediated lipid peroxidation has the potential to result in significant subcellular damage (Zimmerman et al., 1999), the extent to which it contributes to DON-related hepatic histopathogenesis is unknown. Tiemann et al. (2006b; 2008a, 2008b) suggested that a significant increase in iron staining due to hemosiderin deposition in the livers of pigs fed diets containing 3.1 to 9.6 ppm DON could potentially lead to oxidative stress-induced cell damage. Indeed, there is considerable evidence that excess tissue content of iron can result in free radical production and peroxidative injury. Ultimately, damage to intracellular organelles may occur (Irving et al., 1988; Bacon and Britton, 1989; Zimmerman et al., 1999). Fatty changes and cytoplasmic vacuolation of the livers of some fish species fed DON-contaminated diets is in agreement with the conclusions of histological and ultrastructural work conducted in swine (Tiemann et al., 2006b, 2008a; Grenier et al., 2011). Hyperaemia (increased arteriolar blood flow to a site) is usually associated with
vascular dilation due to localized release of inflammatory mediators (Mitchell and Cotran, 2003; Mumford et al., 2007; Damjanov, 2012). It is possible, then, that the appearance of hyperaemia in the livers of carp fed contaminated diets is related to the proinflammatory response associated with acute, low dose exposure to DON (Pietsch et al., 2014b; Pietsch and Burkhardt-Holm, 2015). Acute inflammation may likewise lead to increased vascular permeability culminating in edema. An alternative, noninflammatory cause of edema is reduced plasma osmotic pressure due to hypoalbuminemia, a mechanism mutually compatible with the well-known inhibitory effect of DON on protein synthesis (Mitchell and Cotran, 2003; Mumford et al., 2007; Damjanov, 2012). Interestingly, Pietsch et al. (2014b) reported decreased serum albumin concentration in carp fed diets containing 0.62 or 0.95 ppm DON (Table 2.4). This may explain the incidence of edema in rainbow trout and red tilapia exposed to DON (Hooft et al., 2011; Tola et al., 2015).

2.6.3 – Oxidative stress and cytotoxicity

Although the results of studies conducted to date are somewhat inconsistent, DON does appear to influence various indicators of cellular oxidative status in fish and fish cell lines (Table 2.7). Pietsch et al. (2014a) reported increased SOD and CAT activities in erythrocyte lysates obtained from carp fed a diet containing 0.35 ppm DON compared to fish fed diets containing 0.62 and 0.95 ppm DON. Based on changes in erythrocyte morphology, the authors suggested that cytotoxicity may have prevented an antioxidative response in fish fed the medium- and high-dose diets (Table 2.4). This is in agreement with the hemolytic capacity of DON and T-2 toxin in rat erythrocytes and the protective effect of non-enzymatic free radical scavengers.
including mannitol, glutathione, ascorbic acid, α-tocopherol and histidine (Segal et al., 1983; Rizzo et al., 1992).

Despite no effect on endogenous erythrocyte antioxidant activities, similar sized carp fed the diet containing 0.95 ppm DON experienced decreased SOD, GPx and GR activities in liver tissue homogenates and increased lipid peroxidation in liver, head kidney and spleen (Pietsch et al., 2014b; Pietsch and Burkhardt-Holm, 2015). Likewise, Šišperová et al. (2015) described decreased GR activity in the gills and kidney and decreased GPx activity in the kidney of rainbow trout fed a diet containing 2 ppm DON for 32 days. These changes were accompanied by decreased CAT activity in the kidney and liver, but no effect on renal or hepatic SOD expression was observed (Šišperová et al., 2015). In contrast, Sanden et al. (2012) reported a significant linear increase in liver SOD expression in zebrafish fed six diets containing graded levels of purified DON up to 3 ppm for 45 days. Discrepancies in SOD enzyme activities and mRNA levels across studies could be attributed to several factors including species-, age-, dose- and duration-related differences and post-transcriptional modification. Nonetheless, decreased GPx and GR activities in both rainbow trout and carp suggest an inefficient regeneration or exhaustion of GSH. This, in turn, may be related to increased demand on antioxidant systems posed by DON-induced ROS production.

Sustained perturbation of oxidative balance can significantly impair cell function and may result in cell death (Chandra et al., 2000). Consequently, cytotoxicity assays are frequently conducted in parallel with measures of oxidative stress. Most notably, cell viability is assessed by neutral red (NR) uptake and the MTT assay. The NR assay is based on the uptake of NR and its accumulation in the lysosomes of viable, uninjured cells. The MTT assay, in comparison, is
based on the reduction of a soluble yellow tetrazolium salt to a purple insoluble formazan product via mitochondrial succinate dehydrogenase. High concentrations of DON (800-3000 ng/mL) decreased NR uptake (cell viability) in rainbow trout liver (RTL-W1), gill (RTgill W-1) and epithelial gonadal (RT EQ clone 8) cells and in salmon head kidney (SHK-1) and carp brain (CCB). However, significantly increased NR uptake was observed in RTL W-1, RT EQ clone 8 and CCB cells following incubation with low concentrations of DON (25-50 ng/mL). A pronounced low-dose stimulatory effect and high-dose inhibitory effect of DON on metabolic activity determined using MTT conversion was also apparent in RTgill cells (Table 2.7; (Pietsch et al., 2011). A similar biphasic response of DNA synthesis (measured by BrdU incorporation) was reported in intestinal porcine epithelial cells incubated with DON suggesting that different, unknown mechanisms are responsible for mediating low- and high-dose toxicity in both fish and mammalian cells (Diesing et al., 2011).

Use of complimentary cytotoxicity assays in different models has been essential in delineating cell- and species-specific responses to DON. For example, increased MTT conversion in RT EQ clone 8 cells at all tested concentrations of DON (25-3000 ng/mL) is in contrast to a distinct biphasic effect of DON on NR uptake in this cell type and consistent with cytotoxicity related to altered cell membrane permeability rather than mitochondrial damage. Regardless of these mechanistic implications, the results of both NR uptake and MTT conversion suggested that rainbow trout cells are more sensitive to the cytotoxic effects of DON than CCB and SHK-1 cells \textit{in vitro} (Pietsch et al., 2011). However, NR uptake was significantly reduced in head and trunk kidney leukocytes obtained from carp fed 0.35-0.95 ppm DON for 4 weeks and 0.95 ppm DON for 56 days, respectively (Pietsch et al., 2014a, 2015).
Furthermore, lactate dehydrogenase activity (LDH), an indicator of cellular damage, was significantly increased in cultures of primary carp hepatocytes after incubation with 250 or 500 ng/mL for 4 hours (He et al., 2010). These concentrations are within the range of those associated with changes in cell membrane integrity and mitochondrial function in rainbow trout liver, gill and epithelial gonadal cells (Pietsch et al., 2011). Therefore, the cytotoxic effects of DON are likely species- and organ-specific and highly contingent on the endpoint investigated. The relationship of oxidative stress and cytotoxicity in fish and fish cell lines has not yet been elucidated.

2.6.4 – Immune response and disease resistance

Recent studies have provided interesting insight into the immunomodulatory capacity of DON in fish, particularly within the context of the host-pathogen relationship (Table 2.6). Mortality of rainbow trout fed diets containing 4.1 or 5.9 ppm DON from naturally contaminated corn or 6.4 ppm purified DON was significantly reduced compared to fish fed a control diet following experimental infection with Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (Ryerse et al., 2014, 2015). In agreement, feeding diets naturally contaminated with 5.5, 7.7 or 8.8 ppm DON resulted in a similar reduction in mortality of channel catfish fingerlings challenged with Edwardsiella ictaluri compared to groups fed a diet containing 3.3 ppm DON or a control diet (Manning et al., 2014). This finding is in direct contrast to earlier work demonstrating increased mortality of channel catfish fingerlings of a similar size fed diets containing T-2 toxin (1 or 2 ppm) or OTA (4 ppm) for 6 weeks prior to challenge with the same pathogenic bacteria (Manning et al., 2005).
The mechanism(s) by which DON improves survival in fish exposed to infectious agents remains unclear. Ryerse et al. (2014) proposed that reduced feed intake associated with DON may be a contributing factor based on published and anecdotal evidence supporting feed restriction as an effective management strategy in the control of infectious disease outbreaks in several species. However, these authors demonstrated significantly reduced mortality in rainbow trout fed a naturally contaminated diet (5.9 ppm DON) compared to their pair-fed counterparts in response to *F. psychrophilum* infection. Furthermore, antibacterial activity of DON was determined to be an unlikely factor influencing the susceptibility of DON-exposed fish to infection since a concentration of 75 mg/L was required to inhibit the proliferation of *F. psychrophilum* (Ryerse et al., 2015). Feeding DON-contaminated diets had no effect on total leucocyte counts and relatively minor or transient effects on differential white blood cell (WBC) counts in rainbow trout and common carp (Pietsch et al., 2014a, 2015; Ryerse et al., 2015). However, respiratory burst response of head kidney leucocytes determined using real-time luminol-enhanced chemiluminescence was significantly increased in rainbow trout fed diets containing naturally occurring (3.3 ppm) or purified (3.8 ppm) DON compared to pair-fed fish. DON-induced enhanced respiratory burst activity could potentially influence disease susceptibility (Ryerse et al., 2015). In contrast, decreased respiratory burst activity was observed in carp fed diets containing up to 0.95 ppm purified DON using the nitroblue tetrazolium assay (NBT; Pietsch et al., 2014a). These contradictory results may indicate a species- or dose-dependent effect of DON on innate immunity of fish. Alternatively, the discrepancy could be an artifact resulting from the different analytical methods employed. NBT
is incapable of detecting free radicals other than superoxide anion and thus represents only a semiquantitative method (Vera-Jimenez et al., 2013).

Upregulation of genes associated with immunostimulation and immunosuppression has been observed in carp fed a diet containing 0.9 ppm DON (Pietsch et al., 2015). Interestingly, increased expression of genes associated with pro- and anti-inflammatory processes in carp exposed to DON appears to be tissue- and time-specific. Generalized upregulation of both proinflammatory (interferon-gamma [IFN-\(\gamma\)], TNF-\(\alpha\), IL-8, inducible nitric oxide synthase [iNOS]) and anti-inflammatory (IL-10 and two arginase [ARG] isoforms, ARG-1 and ARG-2) markers in the spleen (with the exception of IL-8) and intestine were observed after two weeks in fish fed the diet containing 0.9 ppm DON. Comparatively, only increased expression of ARG-2 was reported in head and trunk kidney tissues at this time point, whereas trunk kidney mRNA levels of IFN-\(\gamma\), TNF-\(\alpha\), IL-8 and ARG-1 were elevated after 26 days of feeding. Longer-term exposure to DON (8 weeks) resulted in increased expression of ARG-1 and ARG-2 transcripts in trunk kidney (Table 2.6; Pietsch et al., 2015). Arginase plays a critical role in the immune response. Macrophages may be classically or alternatively activated contingent on their interaction with Th1-type cytokines (e.g. IFN-\(\gamma\), TNF-\(\alpha\)) or Th2-type cytokines (e.g. IL-10), respectively (Joerink et al., 2006a,b; Munder, 2009). Classically activated macrophages (caMF or M1) utilize L-arginine as a substrate of iNOS to produce L-citrulline and NO, an important mediator of cytotoxicity. Alternatively activated macrophages (aaMF or M2) hydrolyze L-arginine to L-ornithine and urea via arginase. Arginase appears to represent an endogenous immunosuppressive pathway, in part by limiting the availability of L-arginine for antimicrobial NO production. On one hand, the induction of aaMF and related arginase activity is critical in order to minimize immune-related
pathology (i.e. excessive tissue damage due to inflammation). On the other hand, it may be potentially detrimental to the host during an infection by impeding clearance of the relevant microorganism (Munder, 2009). In view of the altered arginase and iNOS expression in carp fed DON-contaminated diets (Pietsch et al., 2015), macrophage polarization and its potential association with improved disease resistance in fish exposed to DON may warrant future consideration.
Table 2.4. Summary of studies or aspects of studies focused on evaluating the effects of DON on a variety of parameters including growth performance, body composition, hematology and selected enzyme activities in fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 (control), 0.8-3.7 ppm</td>
<td>Not specified</td>
<td>Atlantic salmon, IBW=405±31 g</td>
<td>Oral (feed), 15 weeks</td>
<td>↓ in FI and SGR and ↑ in FCR of fish fed diet containing 3.7 ppm vs. control</td>
<td>Döll et al. (2010)</td>
</tr>
<tr>
<td>Control (no AFB&lt;sub&gt;1&lt;/sub&gt; or DON), 0, 0.01 and 0.02 μg/mL AFB&lt;sub&gt;1&lt;/sub&gt;, 0.25 and 0.5 μg/mL DON, all combinations of AFB&lt;sub&gt;1&lt;/sub&gt; + DON</td>
<td>Purified</td>
<td>Primary carp hepatocytes</td>
<td>Incubation Enzyme activity: 4, 8, 16 h Morphology: 24 h</td>
<td>↑ AST of all groups vs. control after 4 h, but no differences after 8 or 16 h; ↑ ALT activity of all groups vs. control after 4, 8 and 16 h with the exception of 0.02 μg/mL AFB&lt;sub&gt;1&lt;/sub&gt; and 0.25 μg/mL DON at 4 h and 0.01 μg/mL AFB&lt;sub&gt;1&lt;/sub&gt; and 0.5 μg/mL DON at 16 h</td>
<td>He et al. (2010)</td>
</tr>
<tr>
<td>0.3 (control and pair-fed), 0.8, 1.4, 2.0, 2.6 ppm</td>
<td>NC corn</td>
<td>Rainbow trout, IBW=24 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>Linear ↓ in WG, TGC, carcass CP content, RN, RE, NRE, ERE and linear and quadratic ↓ in FI and FE; Pair-fed fish had significantly ↑ TGC, FE, CP, RN, NRE, ERE vs. fish fed diet containing 2.6 ppm</td>
<td>Hooft et al. (2011)</td>
</tr>
<tr>
<td>0.3, 1.4, 1.9, 2.0 ppm</td>
<td>NC corn</td>
<td>Rainbow trout, IBW=8.5 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>No effect on ADC of CP and GE</td>
<td>Hooft et al. (2011)</td>
</tr>
<tr>
<td>0.07 (control), 0.31, 0.50, 0.92, 1.15 ppm</td>
<td>NC wheat</td>
<td>Red tilapia (Oreochromis niloticus x O. mossambicus), 4.3 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>Linear ↓ in WG, TGC, FI and FE; Linear and quadratic ↓ in mortality; No effect on hematocrit, plasma ALT and AST activity or HSI</td>
<td>Tola et al. (2015)</td>
</tr>
<tr>
<td>0, 1, 2, 4.9, 7.5, 12.9 ppm</td>
<td>Artificially infected corn</td>
<td>Rainbow trout, IBW=50 g/fish</td>
<td>Oral (feed), 4 weeks</td>
<td>↓ WG (12-92% of control) and FI; ↑ FCR</td>
<td>Woodward et al. (1983)</td>
</tr>
<tr>
<td>0, 19.4, 40.4, 55.3, 84.3, 109.6 ppm</td>
<td>Artificially infected corn</td>
<td>Rainbow trout, IBW &lt; 3 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>Feed refusal of contaminated feeds; Fish fed contaminated diets for first 4 weeks resumed feeding when offered control diet</td>
<td>Woodward et al. (1983)</td>
</tr>
<tr>
<td>0, 1.25, 2.5, 5, 10 ppm</td>
<td>Purified</td>
<td>Channel catfish, IBW=6.8 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>No effect on growth, FI, hematocrit values or liver weights</td>
<td>Manning et al. (2005)</td>
</tr>
<tr>
<td>0, 2.5, 5, 10, 15, 17.5 ppm</td>
<td>NC wheat</td>
<td>Channel catfish, IBW=5 g/fish</td>
<td>Oral (feed), 6 weeks</td>
<td>↓ growth and ↑ FCR of fish fed diets containing 15 or 17.5 ppm DON; No effect on hematocrit values</td>
<td>Manning et al. (2005)</td>
</tr>
</tbody>
</table>
Table 2.4 continued. Summary of studies or aspects of studies focused on evaluating the effects of DON on a variety of parameters including growth performance, body composition, hematology and selected enzyme activities in fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=45-53 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>No effect on WG, hematocrit, haemoglobin or cortisol; ↓ erythrocyte length of fish fed 0.35 ppm vs. control and 0.95 ppm; ↑ erythrocyte nuclei length of fish fed 0.95 vs. 0.35 or 0.62 ppm; ↓ erythrocyte nucleus width of fish fed 0.62 vs. 0.35 ppm</td>
<td>Pietsch et al. (2014a)</td>
</tr>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=30-40 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>↑ LDH activity in head kidney of fish fed DON diets, trunk kidney of fish fed 0.35 and 0.62 ppm and in serum of fish fed 0.95 ppm vs. control after 4 weeks; ↓ LDH activity in head and trunk kidney of fish fed 0.95 ppm vs. control after recovery; ↑ lipid content of fish fed 0.62 and 0.95 ppm vs. control after 4 weeks and ↓ lipid content of fish fed contaminated diets vs. control after recovery; ↑ energy content of fish fed 0.62 ppm vs. control after 4 weeks and ↓ energy content of fish fed 0.95 ppm vs. control after recovery; ↑ hepatic ALT activity in fish fed 0.62 ppm vs. control after 4 weeks and ↓ serum ALT activity in fish fed 0.95 ppm vs. control, 0.35 and 0.62 ppm after 4 weeks; ↑ lactate content of liver of fish fed 0.62 ppm vs. control after recovery; ↑ serum [lactate] of fish fed 0.95 ppm vs. control after 4 weeks and ↑ serum [glucose] and [lactate] of fish fed contaminated diets vs. control after recovery; ↓ serum [albumin] in fish fed 0.62 and 0.95 ppm vs. control after 4 weeks, but no effect on [total protein]; No effect on liver glucose or ascorbate content or AST activity</td>
<td>Pietsch et al. (2014b)</td>
</tr>
</tbody>
</table>
Table 2.4 continued. Summary of studies or aspects of studies focused on evaluating the effects of DON on a variety of parameters including growth performance, body composition, hematology and selected enzyme activities in fish.

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</tr>
</thead>
<tbody>
<tr>
<td>Control (0 ppm), 0.953 ppm</td>
<td>Purified</td>
<td>Carp, 9-12 cm in length</td>
<td>Oral (feed), sampling of livers at 7, 14, 26 and 56 days, fed 2.5% body weight/day</td>
<td>↑ ALT activity of fish fed 0.9 ppm after 14 and 26 days, but no effect on AST or SDH at any time point; ↓ PROD activity of fish fed 0.9 ppm after 7 days; ↓ GST activity (DCNB) of DON group after 7 and 14 days; ↑ GST activity (CDNB) of DON group after 56 days</td>
<td>Pietsch and Burkhardt-Holm (2015)</td>
</tr>
<tr>
<td>0.02 (control), 0.1, 0.5, 1.5, 2.0, 3.0 ppm</td>
<td>Purified</td>
<td>Zebrafish, IBW=25 mg/fish, 30 days post hatch (dph)</td>
<td>Oral (feed), 45 days (performance, GE) or up to 260 days (fecundity)</td>
<td>No effect of diets on fish length, body weight or SGR; Linear ↑ in hepatic gene expression of Cyclin G1; ↑ hepatic gene expression of CYP1A in fish fed 2 ppm vs. control, 0.1, 0.5 and 1.5 ppm; No significant effect of diets on gene expression of PCNA, IL-6R, Caspase 6 or MAPK14a; ↑ fecundity of fish fed 1.5 ppm vs. 0.5 and 3 ppm; No effect of 0.1, 1.5 or 3 ppm on % 5-methylcytosine in embryos from exposed f0 generation; ↑ swimming activity of f1 larvae from f0 parents fed 3 ppm</td>
<td>Sanden et al. (2012)</td>
</tr>
<tr>
<td>Control (0.225 ppm), 2 ppm</td>
<td>Purified</td>
<td>Rainbow trout, 190 ± 30 g/fish</td>
<td>Oral (feed), blood and tissue samples collected after 23 and 32 days, fed 1% body weight/day</td>
<td>No changes in GST activity after 23 days; ↑ GST activity in liver and ↓ GST activity in gills of fish fed 2 ppm after 32 days</td>
<td>Šišperová et al. (2015)</td>
</tr>
<tr>
<td>0.2 (control) or 2 ppm</td>
<td>Purified</td>
<td>Rainbow trout, 1 year of age</td>
<td>Oral (feed), 23 days</td>
<td>No effect on body or liver weights, length, HSI or condition factor; ↓ mean erythrocyte haemoglobin, plasma glucose, cholesterol and NH₃ of fish fed 2 ppm vs. control</td>
<td>Matejova et al. (2014)</td>
</tr>
</tbody>
</table>

IBW, initial body weight; FI, feed intake; SGR, specific growth rate; FCR, feed conversion ratio; AFB₁, aflatoxin B₁; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; NC, naturally contaminated; WG, weight gain; TGC, thermal-unit growth coefficient; CP, crude protein; RN, retained nitrogen; RE, recovered energy; NRE, nitrogen retention efficiency; ERE, energy retention efficiency; FE, feed efficiency; ADC, apparent digestibility coefficient; GE, gross energy; HSI, hepatosomatic index; SDH, sorbitol dehydrogenase; PROD, pentoxyresorufin O-depentylase; GST, glutathione S-transferase; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; CYP1A, cytochrome P450 1A; PCNA, proliferating cell nuclear antigen; IL-6R, interleukin-6 receptor; MAPK14a, mitogen-activated protein kinase 14a
Table 2.5. Summary of aspects of studies focused on evaluating the effects of DON on histological, pathological and/or morphological changes of tissues and organs in fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
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<th>Route of exposure, duration</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0.3 (control and pair-fed), 0.8, 1.4, 2.0, 2.6 ppm</td>
<td>NC corn</td>
<td>Rainbow trout, IBW=24 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>Subcapsular hemorrhage of liver of some fish fed diets containing 1.4, 2 and 2.6 ppm; Morphological changes of liver including subcapsular edema, fatty infiltration and phenotypically altered hepatocytes in some fish fed 1.4 to 2.6 ppm; No pathological changes of distal intestine</td>
<td>Hooft et al. (2011)</td>
</tr>
<tr>
<td>0.2 (control) or 2 ppm</td>
<td>Purified</td>
<td>Rainbow trout, 1 year of age</td>
<td>Oral (feed), 23 days</td>
<td>Severe hyaline droplet degeneration in tubular epithelial cells of caudal kidney of fish fed 2 ppm; Subcapsular hemorrhage of liver of some fish fed 2 ppm</td>
<td>Matejova et al. (2014)</td>
</tr>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=30-40 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>No effect on liver glycogen content; ↑ fat aggregation in liver of fish fed 0.62 and 0.95 ppm vs. control and ↑ hyperaemia in DON-fed groups; ↑ liver lipid content of fish fed 0.95 ppm vs. control after recovery; ↑ dilation of hepatic sinusoids in fish fed 0.35 and 0.62 ppm vs. control; No effect on liver lesions or degree of vacuolization</td>
<td>Pletsch et al. (2014b)</td>
</tr>
<tr>
<td>Control (0 ppm), 0.953 ppm</td>
<td>Purified</td>
<td>Carp, 9-12 cm</td>
<td>Oral (feed), sampling of livers at 7, 14, 26 and 56 days, fed 2.5% body weight/day</td>
<td>↑ hyperaemia, vacuolization and dilation of sinusoids in fish fed 0.9 ppm after 14 days; ↑ fat aggregations and dilation of sinusoids in fish fed 0.9 ppm after 26 days; No difference in these parameters after 7 or 56 days; No difference in lesions at any time point</td>
<td>Pletsch and Burkhardt-Holm (2015)</td>
</tr>
<tr>
<td>0.07 (control), 0.31, 0.50, 0.92, 1.15 ppm</td>
<td>NC wheat</td>
<td>Red tilapia (Oreochromis niloticus x O. mossambicus), 4.3 g/fish</td>
<td>Oral (feed), 8 weeks</td>
<td>Areas of focal necrosis in livers of some fish fed 0.5 and 1.15 ppm; Cytoplasmic vacuolation in livers of some fish fed 0.31 and 0.50 ppm; Subcapsular edema in livers of some fish fed 0.31, 0.50 and 0.92 ppm; No histopathological alterations of liver with ↑ [DON]</td>
<td>Tola et al. (2015)</td>
</tr>
</tbody>
</table>

NC, naturally contaminated; IBW, initial body weight
Table 2.6. Summary of studies or aspects of studies focused on evaluating the effects of DON on immune response and/or disease resistance of fish.

<table>
<thead>
<tr>
<th>DON levels</th>
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<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
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</thead>
<tbody>
<tr>
<td>0, 3.3, 5.5, 7.7, 8.8 ppm</td>
<td>NC corn</td>
<td>Channel catfish, IBW=5.87±0.22 g/fish</td>
<td>Oral (feed), 7 weeks (prior to <em>E. ictaluri</em> challenge), 3 weeks (post-challenge)</td>
<td>Pre-challenge: no effect of contaminated diets on WG, FI or FCR compared to control group; Post-challenge: ↓ mortality of fish fed 5.5, 7.7 and 8.8 ppm vs. control and 3.3 ppm groups</td>
<td>Manning et al. (2014)</td>
</tr>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=45-53 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>No effect on differential blood cell count or arginase activity of trunk or head kidney leucocytes; ↓ respiratory burst activity (RB) of PMA-stimulated trunk kidney leucocytes from fish fed 0.62 and 0.95 ppm vs. control after recovery; ↓ RB of unstimulated head kidney leucocytes from fish fed 0.62 ppm vs. control after treatment; ↓ RB of PMA-stimulated head kidney leucocytes from fish fed contaminated diets vs. control after treatment</td>
<td>Pietsch et al. (2014a)</td>
</tr>
<tr>
<td>Control (0 ppm), 0.953 ppm</td>
<td>Purified</td>
<td>Carp, 9-12 cm in length</td>
<td>Oral (feed), sampling at 7, 14, 26 and 56 days, fed 2.5% body weight/day</td>
<td>No effect on % leucocytes (as % total blood cells); ↑ thrombocytes and ↓ monocytes (% white blood cells) in fish fed 0.9 ppm after 7 days; ↓ lymphocytes and ↑ monocytes and granulocytes in fish fed 0.9 ppm after 14 days; ↑ NO production in LPS-stimulated trunk kidney leucocytes in fish fed 0.9 ppm after 14 days; Gene expression: Head kidney: ↑ ARG-2 after 14 days; Trunk kidney: ↑ ARG-2 after 14 days, ↑ IFN-γ, TNF-α, IL-8 and ARG-1 after 26 days, ↑ ARG-1 and ARG-2 after 56 days; Spleen: ↑ IFN-γ, TNF-α, IL-8, iNOS, IL-10, ARG-1 and ARG-2 after 14 days; Liver: ↑ IL-8, IL-10, ARG-1 and ARG-2 after 26 days; Intestine: ↑ IFN-γ, TNF-α, iNOS, IL-10, ARG-1 and ARG-2 after 14 days; ↑ ARG activity of head kidney leucocytes from fish fed 0.9 ppm after 26 days</td>
<td>Pietsch et al. (2015)</td>
</tr>
</tbody>
</table>
Table 2.6 continued. Summary of studies or aspects of studies focused on evaluating the effects of DON on immune response and/or disease resistance of fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.5, 4.1, 5.9 ppm</td>
<td>NC corn</td>
<td>Rainbow trout, IBW=7.5 g/fish</td>
<td>Oral (feed), 4 weeks (prior to <em>F. psychrophilum</em> infection), 3 weeks (post-infection)</td>
<td>Pre-infection: linear and quadratic ↓ in FI; Post-infection: ↓ mortality of fish fed 4.1 and 5.9 ppm compared to control and pair-fed groups; ↓ mortality of pair-fed vs. control-fed fish; ↓ mortality of fish fed 5.9 ppm vs. pair-fed</td>
<td>Ryerse et al. (2014)</td>
</tr>
<tr>
<td>&lt;0.1 (control and pair-fed), 3.1, 6.4 ppm</td>
<td>Purified</td>
<td>Rainbow trout, IBW=9.5 g/fish</td>
<td>Oral (feed), 4 weeks (prior to <em>F. psychrophilum</em> infection), 3 weeks (post-infection)</td>
<td>Pre-infection: linear ↓ in FI; Post-infection: ↓ mortality of fish fed 6.4 ppm vs. control; ↓ mortality of pair-fed fish vs. control group; No effect of contaminated diets on PCV; No effect of diets on white blood cell populations (as % leucocytes) at day 0, 3, 7 or 14; ↓ lymphocytes and ↑ granulocytes of pair-fed fish vs. 3.1 ppm and ↓ granulocytes of fish fed 3.1 ppm vs. control at day 21</td>
<td>Ryerse et al. (2015)</td>
</tr>
<tr>
<td>&lt;0.1 (control and pair-fed), 3.3, 3.8 ppm</td>
<td>NC corn (3.3 ppm) and purified (3.8 ppm)</td>
<td>Rainbow trout, IBW=103.2 g/fish</td>
<td>Oral (feed), 5 weeks</td>
<td>↓ WG and FI of fish fed 3.3 and 3.8 ppm vs. control; ↓ FI of fish fed purified vs. naturally contaminated; ↑ RB of head kidney leucocytes in fish fed contaminated diets vs. pair-fed group at day 21 and in fish fed purified vs. control diet at day 35</td>
<td>Ryerse et al. (2015)</td>
</tr>
</tbody>
</table>

NC, naturally contaminated; IBW=initial body weight; WG, weight gain; FI, feed intake; FCR, feed conversion ratio; PMA, phorbol myristate acetate (stimulant for production of ROS by leucocytes); ARG-2, arginase 2; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor alpha; IL-8, interleukin-8; ARG-1, arginase 1; iNOS, inducible nitric oxide synthase; IL-10, interleukin-10; PCV, packed-cell volume (hematocrit)
Table 2.7. Summary of studies or aspects of studies focused on evaluating the effects of DON on indicators of oxidative stress and cytotoxicity in fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 0, 0.01 and 0.02 μg/mL AFB&lt;sub&gt;1&lt;/sub&gt;, 0.25 and 0.5 μg/mL DON, all combinations of AFB&lt;sub&gt;1&lt;/sub&gt; + DON</td>
<td>Purified</td>
<td>Primary carp hepatocytes</td>
<td>Incubation, Cytotoxicity: 2, 6, 10, 14, 18, 22 h</td>
<td>0.02 μg/mL AFB&lt;sub&gt;1&lt;/sub&gt; and 0.5 μg/mL DON was the most toxic (88% cell inhibitory rate); ↑ LDH activity of all groups vs. control after 4 h, but no differences after 8 or 16 h;</td>
<td>He et al. (2010)</td>
</tr>
<tr>
<td>25-3000 ng/mL</td>
<td>Purified</td>
<td>Rainbow trout liver (RTL W-1), gill (RTgill W-1) and epithelial gonadal (RT EQ clone 8) cells, salmon head kidney (SHK-1) cells, carp brain (CCB) cells</td>
<td>Incubation, 24h</td>
<td>↑ ROS at 25 and 1000-3000 ng/mL in RT EQ; ↓ ROS at 400-3000 ng/mL in RTL, SHK-1 and CCB, 200 ng/mL in RTgill and 50-1000 ng/mL in RT EQ; ↑ NR uptake in RTL at 50 ng/mL, RT EQ at 25 and 50 ng/mL and CCB at 25 ng/mL; ↓ NR uptake in RTL and RTgill at 800-3000 ng/mL, RT EQ at 200-3000 ng/mL and SHK-1 and CCB at 3000 ng/mL; ↑ MTT conversion in RTgill at 25 and 50 ng/mL, RT EQ at 25-3000 ng/mL and SHK-1 at 200 and 400 ng/mL; ↓ MTT conversion in RTL at 100-3000 ng/mL and RTgill at 800-3000 ng/mL; ↑ PI in RTL at 1000-3000 ng/mL, RTgill at 25, 50 and 800-3000 ng/mL, RT EQ at 50-3000 ng/mL and SHK-1 at 3000 ng/mL; ↓ PI in RTL at 200 and 400 ng/mL, RTgill at 400 ng/mL, SHK-1 at 100 and 400 ng/mL and CCB at 200-400 ng/mL</td>
<td>Pietsch et al. (2011)</td>
</tr>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=45-53 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>↑ SOD activity of fish fed 0.35 ppm vs. control, 0.62 and 0.95 ppm during recovery; ↑ CAT activity of fish fed 0.35 vs. 0.62 and 0.95 ppm; ↓ cell viability (NR uptake) of head kidney cells of fish fed DON</td>
<td>Pietsch et al. (2014a)</td>
</tr>
</tbody>
</table>
Table 2.7 continued. Summary of studies or aspects of studies focused on evaluating the effects of DON on indicators of oxidative stress and cytotoxicity in fish.

<table>
<thead>
<tr>
<th>DON levels</th>
<th>DON source</th>
<th>Species or in vitro model</th>
<th>Route of exposure, duration</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diet (control), 0.35, 0.62, 0.95 ppm</td>
<td>Purified</td>
<td>Carp, IBW=30-40 g/fish, 12-16 cm</td>
<td>Oral (feed), 4 weeks treatment (control and contaminated diets) and 2 weeks recovery (control), fed 2% body weight/day</td>
<td>↑ lipid peroxidation in head kidney, liver and spleen of fish fed 0.95 ppm vs. control after 4 weeks; ↓ lipid peroxidation in trunk kidney of fish fed 0.95 ppm after 4 weeks; ↓ lipid peroxidation in trunk kidney of fish fed DON diets and in liver of fish fed 0.95 ppm after recovery</td>
<td>Pietsch et al. (2014b)</td>
</tr>
<tr>
<td>Control (0 ppm), 0.953 ppm</td>
<td>Purified</td>
<td>Carp, 9-12 cm in length</td>
<td>Oral (feed), sampling of livers at 7, 14, 26 and 56 days, fed 2.5% body weight/day</td>
<td>↓ GR activity of fish fed 0.9 ppm after 26 days; ↓ Se-GPx and SOD activity of fish fed 0.9 ppm after 56 days</td>
<td>Pietsch and Burkhardt-Holm (2015)</td>
</tr>
<tr>
<td>Control (0 ppm), 0.953 ppm</td>
<td>Purified</td>
<td>Carp, 9-12 cm in length</td>
<td>Oral (feed), sampling at 7, 14, 26 and 56 days, fed 2.5% body weight/day</td>
<td>↓ cell viability (NR uptake) in LPS-stimulated and unstimulated trunk kidney leucocytes of fish fed 0.9 ppm after 56 days</td>
<td>Pietsch et al. (2015)</td>
</tr>
<tr>
<td>0.02 (control), 0.1, 0.5, 1.5, 2.0, 3.0 ppm</td>
<td>Purified</td>
<td>Zebrafish, IBW=25 mg/fish, 30 days post hatch (dph)</td>
<td>Oral (feed), 45 days (performance, GE) or up to 260 days (fecundity)</td>
<td>Linear ↑ in hepatic gene expression of CuZn SOD activity</td>
<td>Sanden et al. (2012)</td>
</tr>
<tr>
<td>Control (0.225 ppm), 2 ppm</td>
<td>Purified</td>
<td>Rainbow trout, 190 ± 30 g/fish</td>
<td>Oral (feed), blood and tissue samples collected after 23 and 32 days, fed 1% body weight/day</td>
<td>No difference in gene expression of SOD; ↓ in CAT activity in kidney of fish fed 2 ppm after 23 and 32 days and in liver of fish fed 2 ppm after 23 days; ↑ GR activity in kidney of fish fed 2 ppm after 23 days; ↓ GR activity in gills and kidney after 32 days; ↓ GPx activity in kidney of fish fed 2 ppm after 32 days; No differences in lipid peroxidation or FRAP</td>
<td>Šišperová et al. (2015)</td>
</tr>
</tbody>
</table>

AFB₁, aflatoxin B₁; PI, propidium iodide fluorescence; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; NO, nitric oxide; LPS, lipopolysaccharide; GR, glutathione reductase; Se-GPx, selenium dependent glutathione peroxidase; FRAP, ferric reducing ability of plasma.
2.7 – Mechanisms of DON-induced emesis and anorexia

2.7.1 – Taste aversion and palatability

Early studies aimed at determining the underlying causes of reduced feed intake and feed refusal associated with DON were focused on the potential contribution of adverse taste. Clark et al. (1987) initially demonstrated that a transitory conditioned taste aversion (CTA) to a novel taste (saccharin) could be established in Sprague-Dawley rats by concurrent feeding of diets contaminated with 4 or 8 ppm DON. However, rats showed no preference for an uncontaminated diet compared to a contaminated diet (0.25 to 8 ppm DON) suggesting that palatability was not altered. In agreement, Ossenkopp et al. (1994) later showed that aversion to saccharin was related to the postingestional effects of DON which could be attenuated by lesions to the area postrema (AP), a region of the brainstem involved in chemoreception. Furthermore, no significant differences in feed consumption or weight gain of pigs fed a diet containing 4 ppm DON or administered an equivalent dose via osmotic pump were observed over a 7 day period. Only at a higher concentration (9 ppm DON) was feed intake of pigs fed a contaminated diet significantly less than that of those dosed using an osmotic pump (Prelusky, 1997) indicating that altered perception of feeds contaminated with DON may be species- and/or dose-dependent. Nonetheless, to date, little conclusive evidence has been generated in support of a significant role of taste or palatability in reduced feed intake associated with DON.
2.7.2 – Enteroendocrine and neuroendocrine effects

2.7.2.1 – 5-HT

The contribution of altered neuroendocrine signaling within the gut-brain axis to DON-induced anorexia in animals has received considerable attention. Most notably, numerous studies have focused on 5-hydroxytryptamine (5-HT; serotonin) as a candidate for the mechanistic explanation of DON’s capacity to rapidly suppress feed intake. 5-HT is a well-known mediator of a variety of behaviours including sleep patterns, mood, muscle coordination and feed intake (Leathwood, 1987). The majority of 5-HT (95%) is produced by the enterochromaffin (EC) cells in the gut and released upon stimulation (i.e. ingestion of food or noxious substances). Following release, 5-HT can act peripherally on receptors within the enteric nervous system and the vagus nerve or on the central nervous system directly via circumventricular organs (structures of the brain characterized by lack of a normal blood-brain barrier) such as the AP. Regarding the former mode of action, Fioramonti et al. (1993) demonstrated that DON can inhibit gastric emptying by acting on small intestinal motility and that this effect is mediated through enteric 5-HT\textsubscript{3} receptors. In accordance with this finding, gastric relaxation and/or delayed gastric emptying have been identified as important components of emesis (Andrews and Hawthorn, 1988) and food intake (Hunt, 1980).

The potential involvement of the central serotoninergic system in DON-mediated feed intake reduction has also been reported. Fitzpatrick et al. (1988) found highly significant increases in 5-HT and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in various regions of the brain of rats following oral administration of 2.5 mg DON/kg body weight. In barrows, acute intravenous (i.v.) administration of a much lesser dose (0.25 mg DON/kg body
weight) resulted in an initial increase in hypothalamic 5-HT one hour post-dosing followed by a significant decrease after 8 hours. However, the ratio of 5-HIAA: 5-HT, an index of 5-HT metabolism, remained elevated suggesting enhanced utilization of 5-HT which the authors hypothesized could have anorectic consequences (Prelusky et al., 1992). Similarly, feeding diets naturally contaminated with DON, 15-ADON, ZON and fusaric acid to starter pigs elevated the 5-HIAA: 5-HT ratio in the hypothalamus and pons compared to control and pair-fed animals (Swamy et al., 2002b, 2004). In contrast, broiler chicks fed contaminated diets with a similar mycotoxin profile experienced a linear decrease in the pons 5-HIAA: 5-HT ratio due to a proportionally greater increase in 5-HT relative to 5-HIAA (Swamy et al., 2004). This trend was paralleled by a significant reduction in feed intake and subsequently confirmed in laying hens fed a diet containing 12.1 ppm DON, 0.5 ppm 15-ADON and 0.6 ppm ZON; comparatively, no significant effects on 5-HT, 5-HIAA or 5-HIAA: 5-HT were observed in the pons, hypothalamus or cortex of broiler breeder hens or turkey poults fed diets contaminated with 12.6 or 6.8 ppm DON, respectively (Yegani et al., 2006).

Despite evidence supporting a CNS effect of DON on 5-HT concentration and/or turnover, the origin and mechanism of this apparent increase in serotonergic activity remains unclear. Smith (1992) proposed that hyperaminoacidemia related to the inhibition of protein synthesis following trichothecene exposure results in increased uptake of tryptophan (trp), an essential 5-HT precursor, across the blood-brain barrier. However, Prelusky (1994) failed to demonstrate an effect of DON on plasma concentrations of 5-HT, 5-HIAA or trp in pigs. Conflicting results have also been observed regarding the role of central 5-HT receptors in DON-induced anorexia and emesis. Antagonists specific to 5-HT3, 5-HT1B or 5-HT2C receptors were
unable to attenuate DON-induced anorexia in the mouse (Flannery, 2012). The apparent lack of affinity of radiolabelled DON for different 5-HT receptor subtypes within several regions of the pig brain seems to validate this finding (Prelusky, 1996). Conversely, low doses of selective 5-HT₃ antagonists were highly effective in preventing DON-induced emesis in the pig (Prelusky and Trenholm, 1993). It has been proposed that the increase in central serotoninergic activity associated with DON may be initiated peripherally. In particular, 5-HT released from the intestine may stimulate gastric afferents (5-HT₃ receptors), subsequently relaying signals to the chemoreceptor trigger zone (CTZ), an important component of the emetic center located within the area postrema (Prelusky and Trenholm, 1993; Prelusky et al., 1997).

### 2.7.2.2 – PYY and CCK

More recently, investigators have sought to determine the involvement of the satiety-regulating hormones, peptide tyrosine tyrosine (PYY) and cholecystokinin (CCK), in the initiation of DON-induced anorexia. Both PYY and CCK are produced by enteroendocrine cells in the gastrointestinal tract. PYY is secreted by the L cells of the ileum and colon and can reduce feed intake by increasing the expression of anorexigenic peptides and decreasing the expression of orexigenic peptides within the hypothalamus. Similarly, CCK is a peptide hormone secreted by the I cells of the duodenum that acts on vagal afferent neurons to increase expression of anorexigenic peptides. Robust increases in plasma concentrations of PYY and CCK consistent with the onset and duration of feed refusal were observed in mice following both i.p. and orolingual exposure to 1 and 5 mg DON/ kg body weight. Use of receptor inhibitors suggested a more dominant role of PYY in DON-induced anorexia (Flannery et al., 2012). In agreement,
elevated plasma concentrations of PYY and 5-HT, but not CCK, occurred in mink following i.p. injection of 0.25 mg DON/kg body weight. Significant reductions in the number of emetic events (i.e. retching and vomiting) were observed in mink administered a neuropeptide Y2 receptor antagonist or a 5-HT_{3} receptor antagonist prior to treatment with DON. Animals pretreated with the 5-HT_{3} antagonist also experienced significantly fewer PYY-induced emetic events compared to those administered PYY following treatment with PBS (negative control). When considered together with the kinetics of plasma PYY and 5-HT increases, this finding suggests that PYY may somehow be involved in initiating or potentiating 5-HT-mediated events (Wu et al., 2013). In contrast, acute i.p. exposure to DON had no effect on plasma concentrations of other satiety-regulating hormones in mice including glucagon-like peptide-1 (GLP-1), leptin, amylin, pancreatic polypeptide (PP), gastric inhibitory peptide (GIP) or ghrelin (Flannery et al., 2012).

2.7.3 – Central anorexigenic signals

As alluded to previously, both the hypothalamus and brainstem play important roles in the control of appetite. Extensive reciprocal connections exist between the hypothalamus and the brainstem, particularly via the nucleus tractus solitarius (NTS). The NTS is in close anatomical proximity to the AP and is therefore able to respond to peripheral circulating signals in addition to receiving vagal afferents from the gastrointestinal tract. Within the hypothalamus, the arcuate nucleus (ARC) is comprised of two primary neuronal populations with opposing effects on appetite: one subpopulation which express the orexigenic proteins neuropeptide Y (NPY) and agouti-related peptide (AgRP) and a second subpopulation which co-
express the anorexigenic proteins pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). Populations of NPY/AgRP and POMC/CART neurons project to several other areas of the hypothalamus including the paraventricular nucleus (PVN), an integration point for neuropeptide signals originating from the ARC and brainstem. POMC is cleaved to produce α-melanocyte stimulating hormone (α-MSH) which acts on melanocortin-4 receptors (MC4R) mainly located in the PVN to suppress food intake. The ARC lies in close proximity to the median eminence, which, like the AP, lacks a complete blood-brain barrier. Consequently, in addition to receiving signals via the vagal-NTS pathway, the ARC may also integrate information directly from circulating peripheral factors (Girardet et al., 2011; Flannery, 2012).

Mice orally gavaged with 12.5 mg DON/kg body weight exhibited a significant increase in the number of c-Fos stained nuclei (an indicator of neuronal activation) in the AP, NTS, PVN and ARC. Oral administration of DON also resulted in increased hypothalamic mRNA expression of POMC, CART and MC4R. Conversely, the expression of NPY and AgRP was unaffected. Interestingly, vagotomy failed to reduce c-Fos expression in the NTS following DON exposure suggesting that the ability of DON to act directly on the central nervous system may be crucial to its anorectic effects (Girardet et al., 2011). Nonetheless, continued research is necessary to delineate the relative importance of peripheral and central mechanisms to DON-induced anorexia.
Figure 2.3. Simplistic representation of one of the possible mechanisms of DON-induced anorexia. DON results in the release of satiety factors from the gut such as 5-HT or PYY, which, in turn, may reduce feed intake by inhibiting gastric emptying and motility and/or altering the balance of orexigenic (NPY/AgRP) and anorexigenic (POMC/CART) peptides with the hypothalamus. Satiety signals reach the hypothalamus via the peripheral vagus-NTS-hypothalamic pathway or centrally via the bloodstream-circumventricular organ-NTS/ARC pathway. Alternatively, DON may exert its effects directly via the circumventricular organs. ARC, arcuate nucleus; PVN, paraventricular nucleus; NTS, nucleus tractus solitarius; ME, median eminence; AP, area postrema. Adapted from Flannery, 2012.
2.8 – Mycotoxin interactions

Commercial feed ingredients are routinely contaminated with multiple mycotoxins. This is partially due to the fact that many fungal species are capable of simultaneously producing more than one mycotoxin (section 2.2). The inclusion of numerous plant-based ingredients can also contribute to the presence of several mycotoxins in a single feed. It is generally acknowledged that concentrations of individual mycotoxins associated with poor animal performance or compromised health status in commercial operations are usually lower than those reported to cause toxic effects in controlled laboratory settings (Trenholm et al., 1983; Smith et al., 1997; CAST, 2003). Moreover, in some cases, purified DON has been shown to be less toxic to animals than DON from naturally contaminated sources when equivalent amounts are fed (Forsyth et al., 1977; Friend et al., 1986b; Trenholm et al., 1994). The implications of toxicological interactions between mycotoxins must therefore be sufficiently considered in studies employing experimental diets formulated with naturally contaminated ingredients.

Interactions between concomitantly occurring mycotoxins are commonly classified as follows: (1) synergistic, in which the effect of the mycotoxin combination is greater than the expected sum of the individual effects of each mycotoxin (e.g. 2+2=10); (2) potentiative, in which one mycotoxin does not result in a toxic effect alone, but rather, potentiates or increases the effect of another mycotoxin (e.g. 0+2=10); (3) additive, in which the effect of the mycotoxin combination is equal to the sum of the individual effects of each mycotoxin (e.g. 2+4=6); (4) less than additive, in which the effect of the mycotoxin combination mainly reflects the effect of only one mycotoxin without additional effect of the other mycotoxin (e.g. 2+4=4) or (5) antagonistic, in which one mycotoxin interferes with the effects of another and the effect of the
mycotoxin combination is less than the effect of the more potent mycotoxin (e.g. 4+0=1; CAST, 2003; Eaton and Gilbert, 2008; Grenier and Oswald, 2011).

The impact of interactive effects between co-occurring mycotoxins on animal performance has been previously reviewed for numerous combinations of two mycotoxins (CAST, 2003; Grenier and Oswald, 2011). For purposes of brevity and relevance to the work presented herein, this section will focus mainly on known or potential interactions between DON and other Fusarium mycotoxins found to most frequently co-occur with DON in naturally contaminated feedstuffs.

2.8.1 – DON and fumonisins

DON and fumonisins (FUM) are the most frequently occurring Fusarium mycotoxins. FUM are produced mainly by F. verticillioides and F. proliferatum and are found predominately in corn and corn-based feeds (Ross et al., 1992). Numerous FUM belonging to three different classes (A, B and C) have been characterized. With regard to toxicity, the B series of FUM (FB), fumonisin B₁ (FB₁), B₂ (FB₂), B₃ (FB₃) and B₄ (FB₄), represent the greatest risk to animal health and productivity (Krska et al., 2007). A multi-year global survey of raw feed ingredients and finished feeds indicated that 54% of more than 10,000 samples analyzed contained detectable concentrations of FUM (averaging 1.6 ppm) reported as the sum of FB₁, FB₂ and FB₃ (Streit et al., 2013; Schatzmayr and Streit, 2013). Of these, FB₁ is the most widely studied and most prevalent, accounting for approximately 70% of the total FUM content of feedstuffs (Krska et al., 2007). Grenier et al. (2011) cited unpublished data indicating that 65% of tested samples of corn harvested from 2004 to 2006 in France were co-contaminated with DON and FB. The
principal mechanism of action of FB₁ is related to its ability to interfere with the de novo synthesis and turnover of sphingolipids, a chemically and functionally diverse group of biomolecules which play critical roles in the regulation of cell survival and differentiation (Ghosh et al., 1997; Merrill et al., 2001). Depending on dose and duration of exposure, FB₁ is associated with a wide range of pathologies including the species-specific conditions equine leukoencephalomalacia (ELEM) and porcine pulmonary edema (PPE; Voss et al., 2007).

Interactive effects between DON and FB have also been reported. Young male pigs fed diets co-contaminated with DON and FB (3.1 ppm DON and 6.5 ppm FB₁ + FB₂) displayed elevated liver lesion scores based on the severity and extent of several histopathological endpoints compared to animals fed mono-contaminated diets (2.8 ppm DON or 5.9 ppm FB₁ + FB₂; Grenier et al., 2011). The authors suggested that this additive effect could be related to increased absorption of FB, otherwise poorly absorbed, in the presence of DON (Grenier et al., 2011; Voss et al., 2007). Indeed, compromised intestinal barrier function evident as reduced expression of tight junction proteins (e.g. claudin, occluding and E-cadherin) and decreased trans-epithelial electrical resistance (TEER) in response to DON has been previously demonstrated (Pinton et al., 2009, 2010; Bracarense et al., 2012). In contrast, an antagonistic effect of FB on DON-induced elevation of specific immunoglobulin A (IgA) was reported (Grenier et al., 2011). Selective upregulation of serum IgA is a well-established immunological consequence of DON exposure (Pestka, 2003). It was suggested that the capacity of FB to counteract the DON-induced increase in IgA is related to the depletion of sphingomyelin, a complex sphingolipid shown to promote IgA production and/or secretion in the large intestine (Furuya et al., 2008). FB also prevented DON-induced jejunal and ileal expression of IL-6.
(Bracarense et al., 2012). Interestingly, IL-6 deficient mice are refractory to DON-induced dysregulation of IgA production and the development of IgA nephropathy (Pestka and Zhou, 2000). The common ability of DON and FB to target MAPKs has been proposed as a potential explanation for their interactive effects on cytokine expression (Pinelli et al., 1999; Zhou et al., 2003a). Taken together, these studies suggest that the co-occurrence of FB and DON in feed likely poses an enhanced risk to animal health compared to individual exposure to either mycotoxin, at least in species sensitive to DON.

2.8.2 – DON and ZON

ZON and its metabolites are non-steroidal, estrogenic mycotoxins capable of competitively binding to estrogen receptors. ZON passively crosses the cell membrane and binds to the cytoplasmic estrogen receptor. The ZON-receptor complex is then rapidly transferred to the nucleus, where it binds to estrogen-responsive elements, thereby activating gene transcription (Fink-Gremmels, 2008). In pigs, symptoms of hyperestrogenism generally appear when the level of ZON in the diet reaches or exceeds 1 ppm (James and Smith, 1982). Adverse effects in sexually mature females related to the consumption of ZON-contaminated feed include decreased fertility, prolonged return to estrus, abortion, ovarian atrophy, reddening of the vulva and prolapsed uterus and rectum (Fink-Gremmels, 2008).

Few studies have investigated the interaction between DON and ZON, particularly with regard to performance parameters of commercially important species. Using semi-purified diets, Forsell et al. (1986) were unable to identify any significant synergistic or antagonistic effects of 10 ppm ZON and 5 ppm DON on weight gain, feed intake, terminal organ weights,
hematology or serum Ig levels of weanling female mice. Earlier work by James and Smith (1982) also indicated a lack of adverse effects of diets containing 10 ppm ZON on growth rate, feed consumption or feed efficiency of gilts despite uterine enlargement. However, other evidence suggests that the dietary co-occurrence of DON and ZON at relatively high concentrations may alter aspects of the immune response in either an additive or antagonistic fashion. Host resistance to *Listeria monocytogenes*, assessed by splenic bacterial counts, was reduced to a greater extent by co-administration of 0.5 ppm DON and 10 ppm ZON than by 0.5 ppm DON alone in B6C3F1 mice following a 2-week feeding period. Conversely, the delayed hypersensitivity response was significantly lessened in mice exposed to feed contaminated with 25 ppm DON for 3 weeks compared to those fed a diet containing 25 ppm DON and 10 ppm ZON (Pestka et al., 1987).

### 2.8.3 – DON and fusaric acid

Fusaric acid (5-butylpicolinic) acid, a phytotoxin produced primarily by *F. moniliforme*, has received particular attention as a contaminant of animal feeds due to its well-established presence in North American cereal grains and its potential synergistic interaction with trichothecene mycotoxins (Smith and Sousadias, 1993). Fusaric acid is a pharmacologically active compound; chemical similarity to tryptophan (trp) enables it to compete for binding sites on blood albumin, thereby raising the levels of free trp in the blood. Consequently, trp concentrations and 5-HT synthesis in the brain are increased (Chaouloff et al., 1986). It is also a potent inhibitor of dopamine β-hydroxylase, a key enzyme in the regulation of the synthesis of norepinephrine (Nagatsu et al., 1970). Behavioural changes including vomiting and lethargy
were associated with a trend toward increased concentrations of trp, 5-HT and 5-HIAA in the hypothalamus of pigs orally dosed with a toxic level (200 mg/kg b.w.) of fusaric acid (Smith and MacDonald, 1991). Smith et al. (1997) were subsequently able to demonstrate a toxicological synergism between DON and fusaric acid in swine using naturally contaminated corn, wheat and barley. A significant linear decrease in body weight gain was observed in 8 kg starter pigs fed diets containing relatively constant levels of DON (0.5, 2.2, 2.4 and 2.5 ppm) and increasing concentrations of fusaric acid (2.9, 12.2, 15.9 and 15.6 ppm).

2.8.4 – DON and type A trichothecenes

T-2 toxin, which may be readily metabolized to HT-2 toxin in vivo, is the most acutely toxic trichothecene mycotoxin produced by Fusarium fungi (Ueno, 1984). T-2 and HT-2 toxins are of particular concern in Northern European regions; nonetheless, their presence has been noted in suspect (i.e. produced during abnormally high rainfall) and overwintered North American grains (Scott et al., 1980; Abramson et al., 1987; Miller and Richardson, 2013). The interaction between DON and T-2 toxin was investigated in broiler chicks fed diets containing 16 ppm DON from naturally contaminated wheat and 4 ppm purified T-2 toxin singly or in combination (Kubena et al., 1989). Decreases in body weight gain and serum cholesterol concentration of birds fed DON and T-2 toxin were attributed to an additive interaction, while less than additive effects predominantly due to T-2 toxin were associated with a significant decrease in serum albumin concentration and serum LDH activity and a significant increase in oral lesion score. In contrast, poorer feed efficiency, increased relative gizzard weight and reduced mean corpuscular volume (MCV) mainly reflected the presence of DON in the co-
contaminated feed (Kubena et al., 1989). Using diets containing graded levels of T-2 toxin (0, 0.4, 0.8, 1.6, 3.2 ppm) with or without 2.5 ppm DON, Friend et al. (1992) were similarly able to attribute decreases in ADG and feed intake of 12-week-old pigs to DON and its less than additive or synergistic interaction with T-2 toxin rather than to T-2 toxin alone.

Human lymphocyte cultures were used to investigate the effects of DON, T-2 toxin and DAS on lymphocyte proliferation and Ig production in vitro. Combinations of DON with T-2 toxin or DAS resulted in a significantly lower inhibition of lymphocyte proliferation than the inhibition produced by the individual mycotoxins (Thuvander et al., 1999). The relevance of this antagonistic effect of DON in combination with type A trichothecenes on lymphocyte proliferation in vivo is unknown. However, increasing dietary levels of T-2 toxin reportedly increased the number of lymphocytes in young pigs, whereas the opposite effect was observed with the addition of DON (Friend et al., 1992).

### 2.8.5 – DON and other type B trichothecenes

DON may be produced by different strains (chemotypes) of *F. graminearum* or related species via either of its acetylated precursors, 15-ADON or 3-ADON (Miller and Richardson, 2013). Similarly, the trichothecene biosynthetic pathway of DON-producing *Fusarium* species can yield nivalenol (NIV; Desjardins, 2006). Traces of these metabolites are frequently found to co-exist in commodities contaminated with DON. Abbas et al. (1986) suggested that the presence of 15-ADON in naturally contaminated corn might account, in part, for feed refusal of swine that could not be solely attributed to DON. However, no significant interactions between DON (6 ppm) and 15-ADON or 3-ADON (2 ppm) were observed for ADG, feed consumption or
feed efficiency of 12 to 15 week-old barrows over a 3-week period. The degree of folding and thickness of the esophageal region of the stomach were generally greater in animals fed the DON-contaminated diet compared to the DON-free diet, but the opposite was observed in the presence of 15-ADON. Therefore, while the performance of pigs does not appear to be markedly affected by the occurrence of 15-ADON or 3-ADON in DON-contaminated feed, changes in stomach morphology may be subject to interactive effects (Rotter et al., 1992).

Gouze et al. (2005) evaluated the effects of individual or combined oral doses of DON and NIV (0.071 or 0.355 mg/kg b.w.) on several parameters including plasma biochemistry and metabolic capacity of C57BL/6 mice. Regardless of the dose of NIV or DON, additive and antagonistic interactions were reported for total serum protein level and ethoxyresorufin O-deethylase (EROD) activity, respectively; however, for other endpoints examined including plasma uric acid or IgA concentrations and pentoxyresorufin O-depentylase (PROD) and GST activities the combined effects were highly dynamic depending on the concentration of each toxin (Gouze et al., 2005; Grenier and Oswald, 2011).

2.8.6 – Masked mycotoxins

Like other xenobiotics (e.g. pesticides), plants are able to metabolize mycotoxins to less toxic metabolites via endogenous detoxification processes (Berthiller et al., 2009a). The resulting group of compounds is comprised of conjugated or masked (extractable, soluble) and bound or hidden (non-extractable, insoluble) mycotoxins. Bound mycotoxins are covalently or non-covalently attached to cell wall components such as polymeric carbohydrate or protein matrices. Masked mycotoxins, in contrast, are generally formed by phase II conjugation to
more polar substances (e.g. glycosides; Berthiller et al., 2009a, 2013). As their name implies, masked mycotoxins elude direct detection by routine chromatographic (e.g. HPLC-MS) and immunochemical-based (e.g. ELISA) analytical techniques for several reasons: changed physiochemical properties, modification of an epitope recognized by antibodies or impaired extraction efficiency due to increased polarity when a less polar solvent is used for the extraction of unaltered mycotoxins (Berthiller et al., 2013). With regard to DON, the formation of its glucose conjugate, deoxynivalenol-3-β-ᴅ-glucoside (D3G), represents an important defense mechanism of plants against *Fusarium*-related diseases (Figure 2.4; Savard, 1991; Sewald et al., 1992; Poppenberger et al., 2003). The relative proportion of D3G to DON in DON-contaminated grains varies significantly, but has been shown to range from an average of 20% to as high as 70% (Berthiller et al., 2009b; Desmarchelier and Seefelder, 2011; De Boevre et al., 2012). In some cases, the concentration of D3G may even exceed that of DON (Kostelanska et al., 2009; Varga et al., 2013). Indirect analytical methods in which samples underwent acidic hydrolysis prior to analysis by conventional methods (i.e. GC-ECD or ELISA) increased DON concentrations of barley, corn and wheat by 9-88%, 8-70% and 7-75%, respectively (Zhou et al., 2007; Tran and Smith, 2011).

Until recently, little information concerning the *in vivo* toxicity of D3G was available. Based on *in vitro* models and initial work conducted on a masked form of ZON (zearalenone 4-β-ᴅ-glucopyranoside, Z4G), it was widely hypothesized that D3G could be hydrolyzed to its parent compound in the gastrointestinal tract of mammals, thereby increasing the total DON load (Figure 2.4; (Gareis et al., 1990; Schneweis et al., 2002; Berthiller, 2011; Dall’Erta et al., 2013). Indeed, Nagl et al. (2012, 2014) were able to convincingly demonstrate the nearly
complete hydrolysis of purified D3G in both rats and pigs. However, the oral bioavailability of D3G was reduced by up to a factor of two compared to DON, indicating that it may be of considerably lower toxicological relevance than its native form. Poppenberger et al. (2003) showed that D3G inhibits protein biosynthesis to a far lower extent than DON in wheat ribosomes, but the related consequences have not yet been investigated in mammalian systems. The bioavailability of D3G may also be increased after chronic feed-borne exposure, as has been observed for DON (Goyarts and Dänicke, 2006). Therefore, it remains important to consider the possible biological consequences of this metabolite originating from naturally contaminated feed ingredients. It is also important to note that, in addition to glucose conjugates, limitations exist in terms of accurate quantification of routinely studied mycotoxins in feedstuffs. For example, differences in the detection limits and accuracy of different analytical techniques (e.g. ELISA and HPLC) as well as inter-laboratory variation may contribute to differences between studies (Krska et al., 2001).

2.8.7 – Effect of Fusarium mycotoxin interactions on fish

There currently exists a paucity of information regarding the impact of interactions among Fusarium mycotoxins on fish health and performance. Yildirim et al. (2000) investigated the effects of FB₁ and moniliformin (MON) fed singly or in combination on growth, histopathology and various biochemical parameters of young channel catfish. Feeding diets containing equivalent, relatively high concentrations of FB₁ and MON (40 ppm) to fingerlings for 10 weeks synergistically reduced weight gain and hepatocellular nuclei size and additively increased serum pyruvate concentration, possibly indicating a disruption of mitochondrial
metabolism. Conversely, interactive effects between DON and other *Fusarium* mycotoxins at more realistic levels have not yet been demonstrated in fish. Feed intake of juvenile rainbow trout fed a diet containing 3.8 ppm purified DON was significantly less than that of fish fed a diet containing 3.3 ppm DON (and 0.5 ppm ZON) from naturally contaminated corn; however, there were no differences in weight gain, feed efficiency or respiratory burst activity of head kidney leucocytes suggesting an absence of interactive effects between DON, ZON and other potentially present, but undetectable, metabolites (Ryerse et al., 2015). This finding seems to be indirectly supported by Döll et al. (2010) who did not observe any adverse effects of diets containing up to 0.8 ppm ZON on the growth performance or health of Atlantic salmon. Continued and more extensive efforts are necessary to evaluate the contribution of interactions among *Fusarium* mycotoxins to detrimental effects on fish performance and health, particularly in sensitive species such as Atlantic salmon and rainbow trout and at commercially plausible concentrations. This research is especially needed to facilitate the development of meaningful regulatory and industry guidelines which accurately reflect the risk associated with exposure of farmed fish to mycotoxins.

**2.9 – Toxicokinetics of DON**

**2.9.1 – Absorption and distribution**

Rapid and efficient absorption and extensive distribution of DON in pigs relative to ruminants and poultry is widely discussed as an important determinant of the considerable differences in response to DON among these species (Rotter et al., 1996; Pestka, 2007). Plasma radioactivity levels after intragastric dosing of DON to pigs reached peak concentrations within
15 to 30 minutes of administration, suggesting rapid absorption of the toxin from the gastrointestinal tract (Prelusky et al., 1988). This was later substantiated by Dänicke et al. (2004a) who demonstrated nearly complete absorption of DON from the stomach and proximal small intestine of pigs fed a diet contaminated with 4.2 ppm DON and by Eriksen et al. (2003) who detected DON in the plasma of Swedish Landrace males as early as 20 minutes after ingestion of feed containing 3-ADON. Similarly, maximal plasma and tissue uptake of $^{3}$H-labelled DON occurred within 30 minutes of oral gavage in mice (Azcona-Olivera et al., 1995). The systemic absorption (bioavailability) of DON in pigs based on urinary recovery following consumption of a naturally contaminated feed (5.3 ppm, 0.10 mg/kg b.w./day) or intragastric dosing of $^{14}$C-labelled DON averaged 67 and 68%, respectively (Friend et al., 1986a; Prelusky et al., 1988). Using the ratio of the dose-normalized AUC value (oral vs. i.v. route), Goyarts and Dänicke (2006) observed a similar systemic oral bioavailability of free DON (54%) after acute (one meal) feeding of a diet containing 5.7 ppm DON from contaminated wheat; however, increased bioavailability (89%) occurred following chronic consumption of the same diet over a period of 4 to 6 weeks. Comparatively, limited systemic absorption of DON has been reported in rats (25%; Lake et al., 1987), sheep (6-10%; Prelusky et al., 1985, 1986a, 1987) and poultry including laying hens, broiler breeders and turkeys (1-19%; Prelusky et al., 1986b; Gauvreau, 1991; Osselaere et al., 2013). Interestingly, swine and sheep also show distinct kinetic differences in uptake of DON at the cerebral spinal fluid (CSF). The amount of DON which crossed the blood-brain barrier and reached the CSF was approximately 2.5 times higher in pigs compared to sheep following i.v. dosing (Prelusky and Trenholm, 1991).
Differences in the rates of plasma elimination (elimination half-life, $t_{1/2}$) and volume of distribution ($V_d$) between swine and other species may also explain, in part, the susceptibility of pigs to DON (Rotter et al., 1996; Pestka, 2007). The half-life of a xenobiotic is a function of the intrinsic ability of the body to eliminate the compound and the extent of binding of that compound in blood and extracellular (tissue) space (Prelusky et al., 1988). Although highly dependent on the kinetic model used, the $t_{1/2}$ of DON following intragastric or oral dosing ranged from 5.3 to 7.1 hours in pigs compared to only 2.1 hours in sheep and 44 minutes in turkeys (Prelusky et al., 1985, 1988; Gauvreau, 1991; Dänicke et al., 2004a; Goyarts and Dänicke, 2006). The binding of DON to plasma proteins or red blood cells and plasma clearance capacity for DON are similar in pigs and sheep, thus greater $V_d$ in pigs may contribute to the observed differences in $t_{1/2}$ (Prelusky et al., 1987; Rotter et al., 1996). Indeed, the calculated $V_d$ of DON in pigs (1.34 L/kg) is about eight-fold higher than that of sheep (0.17 L/kg; Prelusky et al., 1987, 1988). More recently, an even higher $V_d$ of free DON was reported in pigs fed acutely (3.98 L/kg) or chronically (2.65 L/kg) with a diet containing 5.7 ppm DON (Goyarts and Dänicke, 2006). The higher $V_d$ of DON in pigs compared to sheep is probably partially due to a greater degree of tissue uptake in the former; however, the effect appears to be very transient. Neither Coppock et al. (1985) nor Prelusky and Trenholm (1991) were able to detect significant residues in tissues of pigs including skeletal muscle 24 hours after i.v. administration of 0.5 or 1.0 mg DON/kg b.w., respectively.
2.9.2 — Gastrointestinal metabolism

The principal DON metabolite produced via intestinal or rumen microbial activity is de-epoxy DON (DOM-1; Figure 2.4). The de-epoxidation of DON to yield DOM-1 is the result of the reduction of the C-12, 13 epoxide group and consequential formation of a double bond at this position. The transformation of DON to DOM-1 is correlated with a loss of cytotoxicity, demonstrated using a cell culture assay (Kollarczik et al., 1994). DOM-1 was first isolated from the excreta of rats orally dosed with DON (Yoshizawa et al., 1983; Côté et al., 1987). Transformation of DON to DOM-1 has since been described in cattle, poultry, swine and fish, although substantial differences in the capacity for rumen/intestinal de-epoxidation and the importance of this metabolic pathway exist among the abovementioned species (King et al., 1984; Côté et al., 1986; He et al., 1992; Guan et al., 2009).

Ruminal metabolism of DON to DOM-1 appears to be extensive at physiologically relevant concentrations of DON. Dänicke et al. (2005) reported that only 15% of ingested DON (89% of which was DOM-1) could be recovered at the proximal duodenum of cows fed DON-contaminated rations (2.5 to 8.1 mg DON/100 kg b.w.) suggesting intensive microbial metabolism in the rumen. Transformation of DON (up to a level equivalent to 10 mg/kg feed) to a less polar compound consistent with DOM-1 was shown to be essentially complete (89%) within 24 hours of incubation with rumen fluid (King et al., 1984). Similar capacity for microbial metabolism of DON is evident in the hindgut of poultry. For example, disappearance of 95 to 98% of purified DON was observed in incubations with cecal and colonic contents obtained from hens (Lun et al., 1988; He et al., 1992). Conversely, incubations with fluid from the small intestine had essentially no effect on the concentration of DON (Lun et al., 1988). The
physiological importance of these findings remains unclear. Presumably, hindgut fermentation would have little impact on the oral bioavailability of DON. The apparent permeability of the jejunal epithelium of chickens to DON is comparable to that of other (more sensitive) species (Awad et al., 2007; Maresca, 2013). Furthermore, high activity of $^{14}$C-labelled DON was observed in the liver and bile of laying hens (Prelusky et al., 1986b; Lun et al., 1988). This could indicate that hepatic metabolism plays a greater role than microbial de-epoxidation in the high tolerance of poultry to DON. It has also been proposed that microbial metabolism of DON could be carried out in the foregut by crop-associated bacteria prior to absorption at the small intestine (Maresca, 2013). The biological implications of intestinal de-epoxidation in fish are likewise not well understood at present. Guan et al. (2009) were able to demonstrate complete transformation of DON to DOM-1 following incubation with digesta from brown bullhead catfish in full medium at 15 °C for 96 hours; in contrast, de-epoxidation did not occur when DON was incubated with digesta from other species including pink salmon and brown trout. However, minimal metabolism of DON (< 10% transformation to DOM-1) was observed following incubation with the catfish microbial culture after 48 hours which more realistically reflects the intestinal transit time of digesta in farmed fish (Sveier et al., 1999).

The sensitivity of pigs to DON is believed to be related to inefficient de-epoxidation in the proximal intestine. Goyarts and Dänicke (2006) reported that only 2.5% of DON consumed by pigs exposed to a naturally contaminated diet (5.7 ppm DON) was recovered in the feces, the majority as DOM-1. Similarly, approximately 2% of ingested 3-ADON (2.5 ppm) was found as DON or DOM-1 in the feces of castrated Swedish Landrace males despite evidence of complete de-acetylation of 3-ADON to DON. Comparatively, nearly 50% of the administered
dose was excreted in the urine as DON or conjugated DON (Eriksen et al., 2003). Dänicke et al. (2004c) demonstrated a significant linear correlation between the dietary level of DON and the concentration of DON in the urine. The percentage of DOM-1 in the urine also increased linearly with increasing levels of DON in the diet, but accounted for just 2% of DON intake compared to the urinary excretion of DON which represented 45 to 55% of DON intake. Interestingly, de-epoxidation of DON, 3-ADON and NIV has been observed following incubation (24-48 hours) with cecal, colonic and rectal contents as well as feces of pigs (Kollarczik et al., 1994; Eriksen et al., 2002). However, DOM-1 was not formed after incubation of DON with porcine duodenal or jejunal contents (Kollarczik et al., 1994). This was reflected by an 80% increase in the proportion of DOM-1 to DON plus DOM-1 (DOM-1/DON+DOM-1) from the distal small intestine to the rectum of pigs fed a diet contaminated with 4.2 ppm DON (Dänicke et al., 2004a). Collectively, these studies support the assumption that de-epoxidation in the large intestine does not contribute much to DON detoxification in the pig because the majority of the parent compound is absorbed proximally in the small intestine (Eriksen et al., 2003; Dänicke et al., 2004a).

**2.9.3 – Phase I and II metabolism**

Drug or xenobiotic metabolism can be divided into two main phases, each carried out by unique sets of metabolic enzymes: phase I (nonsynthetic or functionalization reactions) and phase II (synthetic or conjugative reactions). Phase I reactions are broadly categorized into oxidation, reduction, hydrolysis and hydration. Arguably, oxidations catalyzed by the microsomal cytochrome P450-dependent (CYP450) mixed-function oxidase system constitute
the most diverse and important phase I reactions. Generally, phase I enzymatic reactions act to prepare a compound for subsequent metabolism by addition or unmasking of a chemically reactive functional group required for phase II reactions. In some cases, however, drugs or xenobiotics may bypass phase I and undergo phase II metabolism directly. These reactions involve conjugation of a xenobiotic with endogenous substrates (co-factors) such as UDP-glucuronic acid (UDPGA), glutathione, sulfate or glycine, usually resulting in the formation of water-soluble metabolites which can be more easily excreted in bile, urine or alternatively, through the gills in fish (Gibson and Skett, 2001; Brenner and Stevens, 2010). Some examples of phase II conjugation reactions are provided in Table 2.8.

The role of phase I metabolism in DON detoxification remains somewhat undefined despite evidence of extensive CYP450 involvement in the metabolism of the closely related type A trichothecene T-2 toxin (Wu et al., 2014). Côté et al. (1987) were unable to demonstrate an increase in the rate of NADPH oxidation in viable rat liver microsomes incubated with 1 μmol/mL DON or DOM-1 suggesting a lack of CYP450-dependent metabolism of DON and its de-epoxide metabolite. Morrissey et al. (1985) reached a similar conclusion based on their observation of no change in the hepatic microsomal CYP450 content of rats fed a diet containing 20 ppm purified DON for 90 days. In contrast, increased hepatic expression of CYP50 2B1/2B2 was significantly correlated with an increase in pentoxyresorufin O-depentylase activity (PROD) in mice receiving 0.071 or 0.355 mg DON/kg b.w. (0.5 and 2.3 mg DON/kg feed, respectively) three days per week for a period of four weeks. PROD activity was also significantly increased in animals administered a lower dose of 0.014 mg DON/kg b.w. (0.1 mg DON/kg feed); however, the expression of hepatic CYP450 1A1/1A2 determined by Western
blot analysis and the corresponding activities of ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) remained unchanged in mice receiving up to 1.774 mg DON/kg b.w., the equivalent of approximately 11.7 mg DON/kg feed (Gouze et al., 2006). This result is yet again different from that of Sanden et al. (2012) who found a significant three-fold increase in the liver CYP450 1A mRNA level of zebrafish fed a diet containing 2.0 ppm DON compared to fish in the control, 0.1, 0.5 and 1.5 ppm DON groups. Interestingly, no significant effect on hepatic CYP450 1A was observed in zebrafish fed the most highly contaminated diet containing 3.0 ppm DON. The discrepancies between studies described here may be related to species- or dose-specific effects of DON on hepatic phase I metabolic capacity. Gouze et al. (2006) reported no significant effect of the highest dose of DON used in their study (1.774 mg/kg b.w.) on CYP450 2B protein level or PROD activity despite significant increases in both parameters at intermediary concentrations (0.071 or 0.355 mg DON/kg b.w.) as previously described. The specificity of the analytical approaches used (e.g. determination of total microsomal CYP450 content by spectrophotometry vs. isoenzyme expression vs. catalytic activity) may also contribute substantially to the different conclusions regarding CYP450 metabolism of DON.

The extent of glucuronide conjugation has been routinely assessed by measurement of the native toxin in biological samples after enzymatic hydrolysis with β-glucuronidase (Côté et al., 1986; Prelusky et al., 1986a; Eriksen et al., 2003; Meky et al., 2003; Dänicke et al., 2004b, 2005; Goyarts and Dänicke, 2006). More recently, advanced analytical methods, namely liquid chromatography-tandem mass spectrometry (LC-MS/MS), and synthesis of analytical standards have enabled the identification and quantification of specific DON-GlcA isomers in a one-step
procedure (Warth et al., 2011, 2012a, 2012b; Fruhmann et al., 2012). Maul et al. (2012) investigated the hepatic glucuronidation pattern of DON in several species. Although all microsomal fractions showed capacity for the formation of DON-3-\(\beta\)-d-O-glucuronide (DON-3-GlcA), glucuronidation activity was species-dependent: bovine > rat > carp > trout > porcine > human > chicken. More specifically, glucuronidation activity with regard to the formation of DON-3-GlcA varied from 525 pmol/min/mg protein in bovine liver microsomes to just 0.1 pmol/min/mg protein in chicken liver microsomes. The glucuronidation activities for DON-3-GlcA formation were 14 and 5.8 pmol/min/mg protein in carp and trout liver microsomes, respectively. In addition to DON-3-GlcA, human microsomes formed a second isomer assumed to be DON-15-\(\beta\)-d-O-glucuronide (DON-15-GlcA). Indeed, Warth et al. (2012a, 2013) determined that DON-15-GlcA is the predominant conjugate in human urine, accounting for approximately 75% of total DON-GlcA. UDPGT activity related to a peak tentatively designated DON-7-\(\beta\)-d-O-glucuronide (DON-7-GlcA) was semi-quantitatively ranked as low for carp, human and rat liver microsomes, considerable for bovine and trout liver microsomes and was not detected in porcine or chicken liver microsomes (Maul et al., 2012). However, in-depth structural characterization of DON-GlcAs using nuclear magnetic resonance (NMR) spectroscopy indicated that conjugation at the C-8 position, rather than the C-7 position, is favoured in human liver microsomes (Uhlig et al., 2013). The metabolism of DON to its species-specific glucuronide conjugates is depicted in Figure 2.4.

The lack of glucuronidation in chicken liver microsomes described by Maul et al. (2012) appears somewhat counterintuitive given the high tolerance of this species to DON. Nonetheless, sulfate metabolites were recently identified in chicken and rat excreta following
oral administration of $^3$H-DON. DON-10-sulfonate and DOM-1-10 sulfonate were formed in rats, whereas DON-3-sulfate accounted for about 89% of the administered dose in broiler chickens (Wan et al., 2014; Figure 2.4). Previously, Prelusky et al. (1987) also demonstrated that a minor amount (2%) of the recovered $^{14}$C-DON dose in sheep urine was comprised of a DON-sulfate conjugate. Therefore, it seems plausible that intestinal de-epoxidation and sulfation, rather than glucuronidation, may be responsible for the resistance of chickens to DON.

Glutathione (GSH) conjugation may also be involved in the detoxification of DON in different species. Hepatic glutathione S-transferase (GST) activities measured using the generic substrates 1,2-dichloro-4-nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB) were significantly increased in mice administered 12 oral doses of 0.071 mg DON/kg b.w. or 0.355 mg DON/kg b.w. over a four week period. The increase in GST activity was paralleled by a significant increase in the relative protein expression of the GST π and GST α subfamilies in groups receiving 0.071 and 0.355 mg DON/kg b.w. or 0.71 mg DON/kg b.w., respectively. Furthermore, DON was established as a putative substrate of the GST π subfamily (Gouze et al., 2006).
Table 2.8. Types of conjugation reactions and their respective enzymes, co-factors (i.e. endogenous substrates) and functional groups (i.e. site of conjugation; Gibson and Skett, 2001; Liston et al., 2001).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme$^a$</th>
<th>Co-factor$^b$</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucuronidation</td>
<td>UDPGTs</td>
<td>UDPGA</td>
<td>-OH, -COOH, -NH$_2$, -SH</td>
</tr>
<tr>
<td>Glycosidation</td>
<td>UGTs</td>
<td>UDP-glucose</td>
<td>-OH, -COOH, -SH</td>
</tr>
<tr>
<td>Sulfation</td>
<td>SULTs</td>
<td>PAPS</td>
<td>-NH$_2$, -SO$_2$NH$_2$, -OH</td>
</tr>
<tr>
<td>Methylation</td>
<td>MTs</td>
<td>SAM</td>
<td>-OH, -NH$_2$</td>
</tr>
<tr>
<td>$N$-acetylation</td>
<td>NATs</td>
<td>Acetyl-CoA</td>
<td>-NH$_2$, -SO$_2$NH$_2$, -OH</td>
</tr>
<tr>
<td>Amino acid conjugation</td>
<td>NATs</td>
<td>Gly, Orn, Arg, Tau</td>
<td>-COOH</td>
</tr>
<tr>
<td>Glutathione conjugation</td>
<td>GSTs</td>
<td>GSH</td>
<td>Epoxide, organic halide</td>
</tr>
</tbody>
</table>

$^a$UDPGTs, UDP-glucuronosyltransferases; UGTs, UDP-glycosyltransferases; SULTs, sulfotransferases; MTs, methyltransferases; NATs, $N$-acetyltransferases; GSTs, glutathione S-transferases

$^b$UDPGA, UDP-glucuronic acid; PAPS, 3’-phosphoadenosine-5’-phosphosulfate; SAM, S-adenosyl-L-methionine; Gly, glycine; Orn, ornithine; Arg, arginine; Tau, taurine; GSH, glutathione
Figure 2.4. Phase II metabolism of DON in various species. D3G, DON-3-β-D-glucoside; GlcA, β-D-glucuronide. DOM-1-GlcA and DOM-1-10-sulfonate not shown. Adapted from Wu et al., 2014. Generated using ChemDoodle® version 8.0.1.
2.10 – Nutritional regulation of glucuronidation

2.10.1 – UDPGA and carbohydrate metabolism

The uronic acid pathway, which results in the generation of UDP-glucuronate (anionic/physiologically active form of UDPGA), is closely related to carbohydrate metabolism (Engelking, 2015). Glycolysis is the major route of glucose metabolism and consists of a progressive oxidation of one molecule of glucose into two molecules of pyruvate, accompanied by the net conversion of two molecules of ADP to two molecules of ATP (Figure 2.5; Bender, 2009a; Horton et al., 2002). Glucose derived from dietary carbohydrates is initially metabolized to glucose 6-phosphate by hexokinases. Glucose 6-phosphate may then enter glycolysis. Alternatively, it may be oxidized via the pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt (HMP), or the uronic acid pathway. In the uronic acid pathway, glucose 6-phosphate is initially converted to glucose 1-phosphate, which subsequently reacts with uridine triphosphate (UTP) to form UDP-glucose (Horton et al., 2002; Wamelink et al., 2008; Bender, 2009b). Rapid catabolism of pyrophosphate (PPI) makes this reaction essentially irreversible (Reinke et al., 1994). UDP-glucose is oxidized by an NAD⁺-dependent dehydrogenase to yield UDP-glucuronate (Figure 2.5). UDP-glucuronate, in turn, can act as a co-factor in glucuronidation or be further reduced, resulting in the synthesis of ascorbate (in some species) and ultimately, the production of xylulose 5-phosphate, an intermediate of the PPP (Bender, 2009b; Wamelink et al., 2008).

In addition to glycolysis and the PPP, the uronic acid pathway is connected to glycogenesis (glycogen synthesis), glycogenolysis (glycogen breakdown) and gluconeogenesis (the biosynthesis of glucose from non-carbohydrate precursors). The precursor of UDP-
glucuronate, UDP-glucose, is also the substrate for glycogenesis; that is, glycogenesis is an alternative route to the uronic acid pathway for the utilization of UDP-glucose (Figure 2.5). The addition of the glucose residue from UDP-glucose to the non-reducing end of a pre-existing glycogen primer is catalyzed by glycogen synthase. Glycogen phosphorylase, in contrast, catalyzes the major reaction of glycogenolysis in vertebrates: the sequential removal of glucose from glycogen chains resulting in the production of glucose 1-phosphate (Figure 2.5). Under conditions in which intracellular stores of glycogen have been depleted or there is an insufficient dietary supply of glucose, gluconeogenesis is critical. The major substrates for gluconeogenesis in monogastric species are glucogenic amino acids, lactate and glycerol derived from the catabolism of triacylglycerols. It is most convenient to consider gluconeogenesis as the reverse of glycolysis with pyruvate as a starting point. Three enzymatic reactions unique to gluconeogenesis bypass the metabolically irreversible reactions of glycolysis (Figure 2.5; Horton et al., 2002).

2.10.2 – Carbohydrate metabolism and glucuronidation

Co-factor supply plays an important role in the regulation of mixed-function oxidation (phase I) and glucuronidation (phase II). Several studies have demonstrated a direct correlation between the rate of glucuronidation and the intracellular concentrations of UDPGA (Reinke et al., 1981; Singh and Schwarz, 1981; Aw and Jones, 1984). In addition, the $K_m$ values of UDPGTs for UDPGA have been reported to be of the same order of magnitude as the concentration of UDPGA in rat liver (Bock et al., 1973; Falany and Tephly, 1983). It follows, then, that changes in cellular metabolism which increase or decrease the availability of UDPGA can potentially affect
the rate of glucuronidation (Reinke et al., 1994). Carbohydrate metabolism, in particular, is cited as a critical factor involved in the regulation of glucuronidation (Thurman and Kauffman, 1980; Reinke et al., 1994).

Early investigations regarding the connection between carbohydrate metabolism and co-factor supply for hepatic drug metabolism indicated that UDPGA was more likely derived from carbohydrate stores (i.e. glycogen) or from gluconeogenic amino acids, especially in instances where the supply of glycogen was insufficient, than from glucose (Quick, 1926; Dziewiatkowski and Lewis, 1944). Later, Bánhegyi et al. (1988) convincingly demonstrated that UDPGA for glucuronidation originates predominantly from glycogen, while the generation of NADPH necessary for mixed-function oxidation is related to exogenous glucose uptake and/or gluconeogenesis. In fact, both glycogenolysis and glucuronidation were inhibited in isolated rat hepatocytes when the rate of glycogenesis was stimulated with high amounts of glucose, fructose or insulin (Bánhegyi et al., 1988, 1991). These observations were supported by studies investigating the rate of p-nitrophenol (p-NP) glucuronidation in perfused livers obtained from rats exposed to different feeding strategies: unrestricted access to feed (i.e. fed), fasted for 24 hours prior to perfusion experiments (i.e. fasted) or fasted for 48 hours followed by free access to chow for 24 hours immediately prior to perfusion (i.e. fasted-refed). Maximal rates of glucuronidation were well-correlated with the hepatic contents of glycogen, UDP-glucose and UDPGA (Reinke et al., 1979, 1981). Rapid depletion of hepatic glycogen stores associated with glucuronidation has been observed in rodents administered hepatotoxic doses (400-1000 mg/kg b.w.) of acetaminophen (Hinson et al., 1983; Price et al., 1987; Price and Jollow, 1988). Provision of glucose or gluconeogenic substrates to glycogen depleted fasted rats following
administration of acetaminophen (700 mg/kg b.w.) failed to increase UDP-glucose or UDPGA concentrations and did not increase rates of acetaminophen glucuronidation or protect against acetaminophen-induced liver damage (Price and Jollow, 1989). Interestingly, increased glucuronidation of p-NP and resistance to acetaminophen-induced hepatotoxicity have been demonstrated in male diabetic rats. Enhanced glucuronidation in the diabetic state was primarily attributed to an accelerated rate of UDPGA formation from UDP-glucose as a result of limited conversion of the latter to glycogen. Treatment of diabetic animals with insulin returned glucuronidation to control rates (Eacho et al., 1981; Price and Jollow, 1982).

Marked alterations in drug metabolism (more specifically, oxidation and glucuronidation) can be achieved by manipulating the nutrient composition of the diet. Several clinical studies investigating the relationship between drug metabolism and the dietary carbohydrate to protein ratio have reported increased oxidation (phase I metabolism) in individuals consuming a high protein, low carbohydrate diet as opposed to an isocaloric low protein, high carbohydrate diet (Alvares et al., 1976; Kappas et al., 1976; Feldman et al., 1980; Anderson et al., 1984; Juan et al., 1986; Fagan et al., 1987). Conversely, Sonne et al. (1989) observed a significant decrease in the clearance of oxazepam by glucuronidation and a corresponding increase in its t1/2 in human subjects consuming a low calorie diet sufficient in protein (52.7 g/day), but low in carbohydrates (25.7 g/day). In agreement, increased urinary recoveries of acetaminophen glucuronide and oxazepam glucuronide (14 and 32%, respectively) were observed in healthy male subjects consuming a low protein, high carbohydrate (10% protein, 70% carbohydrates) diet compared to a high protein, low carbohydrate (44% protein, 35% carbohydrates) diet (Pantuck et al., 1991). These findings
highlight the close connection between intermediary metabolism and pathways of drug or xenobiotic metabolism and the potential for nutritional strategies to enhance the capacity for glucuronidation.
Figure 2.5. Pathways of glucose metabolism including glycolysis, gluconeogenesis (red arrows), glycogenesis, glycogenolysis and the part of the uronic acid pathway pertaining to the synthesis of UDP-glucuronate. Adapted from Wamelink et al., 2008; Bender, 2009a,b,c; NRC, 2011.
2.11 – Anti-mycotoxin feed additives

Several pre- and post-harvest mycotoxin mitigation strategies have been investigated in order to prevent or limit mycotoxin contamination of foods and feeds and the development of mycotoxicoses in production species. Preventative approaches are primarily those related to good agricultural practices as well as the use of genetically resistant cultivars, fungicides and bio-competitive exclusion (i.e. inoculation of crops with non-mycotoxin producing fungi or antagonistic bacteria and yeast). Post-harvest strategies for mycotoxin decontamination of crops may include physical and chemical methods such as mechanical separation, density segregation, washing, thermal inactivation, irradiation, solvent extraction, ammoniation and ozonization; however, in many cases, these approaches are not practical (e.g. labor intensive) or cost-effective, can alter the nutritive value of the feedstuff and/or result in the production of toxic by-products (Moss, 1991; Marquardt, 1996; Munkvold, 2003; Kabak et al, 2006; Jouany, 2007). Consequently, anti-mycotoxin functional feed additives have found widespread use in animal nutrition. Commercially available mycotoxin-detoxifying agents are broadly divided into two categories: adsorbing agents and biotransforming agents (Boudergue et al., 2009).

2.11.1 – Mycotoxin-adsorbing agents

Mycotoxin-adsorbing agents are non-nutritive, indigestible large molecular weight compounds capable of binding mycotoxins in contaminated feeds without undergoing dissociation in the gastrointestinal tract of the animal, thereby preventing absorption and distribution of the toxin(s) to the blood and target organs and enabling elimination of the toxin-adsorbent complex in the excreta (Boudergue et al., 2009). Ideally, effective adsorbents should
exhibit several key characteristics: high adsorption capacity, high mycotoxin affinity, no or low desorption of the already adsorbed mycotoxin, no or low adsorption of essential nutrients, absence of toxicity of the binding material itself, low inclusion rate in the feed, rapid and uniform dispersion in the feed during mixing, heat stability and high stability over a wide pH range (Schuh, 2010).

Mycotoxin-adsorbing agents include both inorganic (e.g. silica-based) compounds and organic (e.g. carbon-based) polymers (Kabak et al., 2006; Boudergue et al., 2009). Clay minerals, in particular aluminosilicates (e.g. bentonites, montmorillonites, zeolites and hydrated sodium calcium aluminosilicate; HSCAS), represent the largest class of mycotoxin adsorbents. In general, these compounds are porous materials consisting of a layered silicate or three-dimensional tetrahedral structure. Most aluminosilicates are recognized as efficient binders of the polar aflatoxins, but their efficacy in adsorbing fairly non-polar mycotoxins of practical significance, including fusariotoxins, is extremely limited (Huwig et al., 2001). Moreover, clay silicates may negatively affect the bioavailability of essential nutrients and a high inclusion rate in the feed (5 to 20 g/kg) is usually necessary to attain efficacious adsorption (Moshtagian et al., 1991; Jouany, 2007).

Relative to inorganic binders, organic adsorbents are generally more efficient across a wider range of mycotoxins and are therefore potentially more useful in routinely encountered cases of multi-mycotoxin contaminated feeds. Early studies demonstrated that alfalfa and oat fiber could reduce the adverse effects of ZON exposure in rats and swine (Smith, 1980; James and Smith, 1982; Stangroom and Smith, 1984) and T-2 toxin exposure in rats (Carson and Smith, 1983). Similarly, a significant reduction in the bioavailability of OTA was observed in rats and
pigs fed a diet containing 1 to 2% micronized wheat fibres (Aoudia et al., 2008, 2009). The extracted cell wall fraction of *Saccharomyces cerevisiae* also exhibits considerable ability to bind a number of commonly occurring mycotoxins. A positive correlation between the cell wall β-ᴅ-glucan content of *S. cerevisiae* and its complex-forming efficacy with ZON was subsequently established (Yiannikouris et al., 2004a). Further studies together with molecular modelling revealed the conformational and chemical associations between β-ᴅ-glucans and several mycotoxins (ZON, AFB₁, DON and OTA) including the formation of both hydrogen bonds and van der Waals interactions (Yiannikouris et al., 2004b, 2006).

**2.11.2 – Mycotoxin-biotransforming agents**

Biotransformation involves the degradation or conversion of a parent compound (mycotoxin) into less toxic metabolites in the gastrointestinal tract (Jouany, 2007; Boudergue et al., 2009). Mycotoxin-biotransforming agents may be of bacterial, yeast or fungal origin (Boudergue et al., 2009). Particular success in the development and use of mycotoxin-biotransforming agents has been achieved with regard to the trichothecenes, OTA and ZON (Schatzmayr et al., 2006; Zhou et al., 2008).

The enzymatic biotransformation of DON to its less toxic de-epoxy metabolite, DOM-1, by ruminal and intestinal microorganisms has been extensively investigated within the context of animal metabolism (King et al., 1984; Côté et al., 1986; He et al., 1992; Guan et al., 2009). Binder et al. (1997, 1998) were the first to successfully isolate a novel strain of *Eubacterium* (*Eubacterium* BBSH 797) with epoxidase activity from bovine rumen fluid capable of degrading DON to DOM-1. Fuchs et al. (2002) described complete or partial microbial transformation of
six structurally-related type A trichothecenes (T-2 toxin, HT-2 toxin, T-2 tetraol, T-2 triol, DAS and scirpentriol) to their respective less or non-toxic metabolites following incubation with BBSH 797. Further research within the same institute led to the isolation of a mycotoxin-detoxifying yeast strain characterized as *Trichosporon mycotoxinivorans* (MTV, 115) from the hindgut of a primitive species of termite (*Mastotermes darwiniensis*; Molnar et al., 2004). *T. mycotoxinivorans* detoxifies OTA by cleavage of its peptide bond to yield the non-toxic metabolite ochratoxin alpha (OTα) which lacks the phenylalanine moiety of OTA (Schatzmayr et al., 2006; Pfohl-Leszkowicz and Manderville, 2007). *T. mycotoxinivorans* is also able to detoxify ZON by disrupting its lactone ring structure, producing a metabolite without the estrogenic potency of the parent compound (Schatzmayr et al., 2006).

### 2.11.3 – Efficacy of adsorbing and biotransforming agents in monogastric species

Many research studies have been aimed at evaluating the efficacy of yeast cell wall-derived mycotoxin sequestering agents in different species fed contaminated diets. Raju and Devegowda (2000) concluded that inclusion of 1 g esterified-glucomannan (E-GM) per kg of feed significantly improved weight gain, feed intake and selected hematological and serum biochemical parameters of broiler chickens fed diets containing AFB₁ (0.3 ppm), OTA (2 ppm) and/or T-2 toxin (3 ppm). Swamy et al. (2002a) described similar improvements associated with dietary E-GM inclusion on red blood cell count and hemoglobin, serum uric acid and biliary IgA concentrations of broiler chicks fed a diet containing DON, ZON and FA (9.7, 0.8 and 21.6 ppm, respectively). Conversely, 0.2% inclusion of a yeast cell wall-derived polymeric glucomannan adsorbent (GMA) did not alleviate the detrimental effects of diets naturally
contaminated with *Fusarium* mycotoxins (3.9 ppm DON, 0.4 ppm 15-ADON, 0.4 ppm ZON and 9.9 ppm FA) on body weight, food intake, blood pressure, serum protein content and serum enzyme activities in mature female Beagles (Leung et al., 2007). In partial agreement, addition of GMA (0.05, 0.1 or 0.2%) to naturally contaminated diets containing approximately 5.5 or 5.9 ppm DON, 0.3 or 0.5 ppm 15-ADON, 0.4 ppm ZON and 21 or 28 ppm FA was not effective in preventing mycotoxin-induced reductions in ADG, average daily feed intake or gain to feed ratios in starter pigs (Swamy et al., 2002b, 2003). Nonetheless, dietary GMA supplementation did improve the adverse effects of *Fusarium*-contaminated feeds on brain neurotransmitter concentrations and serum IgA/IgM concentrations in pigs (Swamy et al., 2002b, 2004) and egg-related and metabolic parameters in laying hens (Chowdhury and Smith, 2004). Still, the adsorbent was not efficacious in counteracting a reduction in hepatic fractional protein synthesis rate in birds fed similar diets (Chowdhury and Smith, 2005). These results seem to suggest that the effectiveness of yeast cell wall-derived adsorbents is highly variable and that their efficacy may be species-, mycotoxin-, dose- and endpoint-dependent.

Promising, but contradictory, results have likewise been achieved in monogastric species with the use of mycotoxin-biotransforming agents. Following anaerobic pre-treatment of DON-contaminated corn using large intestinal contents from chickens, He et al. (1993) demonstrated a more than 50% decrease in the DON concentration (4.8 vs. 2.1 ppm) of the finished feed. This translated into significant improvements in weight gain and feed intake of weaned pigs. More recently, the effectiveness of different mycotoxin-biotransforming enzymes has been evaluated. Several studies in particular have focused on the potential efficacy of Mycofix® (BIOMIN Holding GmbH) in preventing mycotoxicoses in monogastric species.
Mycofix® is a proprietary feed additive with epoxidase and lactonase activities based on a combination of clay minerals, *Eubacterium* BBSH 797, MTV and a fumonisins-degrading esterase (FUMzyme®; Pasteiner, 1998; Hofstetter et al., 2006). Inclusion of Mycofix® (0.75 or 1.5 g/kg feed) in diets of growing broilers reportedly prevented the adverse effects of 1 ppm DAS on weight gain and feed intake, but did not alleviate the development of oral lesions or improve growth performance in birds receiving a diet containing 2 ppm DAS (Diaz, 2002). Interestingly, addition of Mycofix® to broiler chick diets (0.25%) attenuated the harmful effects of 10 ppm DON on lymphocyte DNA damage (Awad et al., 2012). Cheng et al. (2006) observed that supplementation of a diet containing 1 ppm DON and 0.25 ppm ZON with 1500 ppm of an unspecified mycotoxin degrading enzyme (MDE) significantly improved the depressive effects of the contaminated feed on ADG, feed intake, FE, total serum protein concentration and macrophage chemotaxis in weanling pigs. On the other hand, animals fed the contaminated diet with or without MDE had significantly elevated serum γ-glutamyltransferase (GGT), ALT and AST activities compared to pigs fed the uncontaminated diet, suggesting that the MDE was not completely effective in preventing the detrimental effects of DON and ZON. These opposing results were supported by the work of Dänicke et al. (2002, 2003, 2004b) who observed little evidence of any beneficial effects of Mycofix® on growth and health indices of broilers, laying hens or pigs fed diets containing *Fusarium*-contaminated wheat or corn. Significant improvements in egg weight and protein digestibility in laying hens fed a diet containing 12 ppm DON and crude fiber digestibility in pigs fed diets containing an average of 2.8 ppm DON were associated with dietary Mycofix® inclusion; however, with the exception of improved fiber digestibility, the positive effects of Mycofix® were determined to be
independent of the mycotoxin contamination of diet (Dänicke et al., 2002, 2004b). In the same sense, The European Food Safety Authority (EFSA, 2005) could not conclusively establish the efficacy of BBSH 797 in poultry and pigs exposed to feed containing *Fusarium* mycotoxins.

To date, much of the published work regarding the efficacy of anti-mycotoxin feed additives in fish has focused on adsorbing agents and AFB$_1$. Smith (1980) documented unexpected improvements in growth and FCR of rainbow trout when clay minerals were added to the diet. This initiated a later study by Ellis et al. (2000) in which the absorption, metabolism and elimination of dietary aflatoxin was traced in rainbow trout consuming a diet containing 20 ppb $^3$H-AFB$_1$ with or without 2% sodium bentonite. The results demonstrated that supplementation of the diet with sodium bentonite prevented intestinal absorption of AFB$_1$, significantly increasing the AFB$_1$ content of the feces and reducing AFB$_1$ recovery from the liver and kidney compared to the positive control group (Ellis et al., 2000). Addition of HSCAS (0.5%), *S. cerevisiae* (0.25%) or E-GM (0.25%) to diets containing 200 ppb AFB$_1$ or intragastric dosing of a clay mineral (Egyptian montmorillonite, EM) in fish administered an aflatoxin precursor, sterigmatocystin (Stg, 1.6 µg/kg b.w.), similarly decreased the respective mycotoxin residues in the muscle of Nile tilapia (Abdel-Wahhab et al., 2005; Selim et al., 2014). Furthermore, administration of EM combined with Stg reduced the number of micronucleated RBCs and decreased the frequency of kidney chromosomal aberrations compared to the group treated with Stg alone, indicating successful prevention of Stg toxicity and clastogenicity (Abdel-Wahhab et al., 2005). In contrast, Zychowski et al. (2013) were not able to substantiate the efficacy of a montmorillonite clay product (NovaSil™) with regard to prevention of the adverse effects of AFB$_1$ (1.5 or 3 ppm) on weight gain, feed efficiency, HSI or hepatic histopathological
lesions of juvenile Nile tilapia. An increase in extracellular superoxide anion production indicated a potential positive effect of NovaSil™ on immunity, but this response occurred regardless of AFB1 contamination of the diet (Zychowski et al., 2013). However, dietary inclusion of HSCAS, *S. cerevisiae* and E-GM counteracted AFB1-induced decreases in growth performance, survival rate, hematological indices and specific immunity as well as AFB1-related increases in serum AST and ALT activities and serum creatinine concentration. In general, HSCAS was more effective than *S. cerevisiae* or E-GM in preventing the detrimental effects of AFB1 on health and performance of tilapia (Selim et al., 2014). Comparable efficacy of a commercial HSCAS (Myco-Ad®) was demonstrated in carp (Agouz and Anwer, 2011).

To my knowledge, Hauptman et al. (2014) have conducted the only published work on the efficacy of a mycotoxin-biotransforming agent in fish. The effect of adding Mycofix® Plus (1 g/kg feed) to diets containing increasing levels (0, 15 and 30%) of Grain Distiller’s Dried Yeasts (GDDY) contaminated with low levels of OTA, DON, ZON, FB1 and FB3 (0.9 ppb, 0.7 ppm, 133 ppb, 0.2 ppm and 0.1 ppm, respectively) was investigated in rainbow trout. Near significant improvements in protein retention efficiency (PRE) and ERE were associated with Mycofix® Plus supplementation and a significant interactive effect of GDDY inclusion level and Mycofix® Plus was observed wherein PRE was increased by Mycofix® Plus supplementation in fish fed the diet containing 30% GDDY. A trend toward a significant improvement in weight gain in fish fed the diets containing Mycofix® Plus regardless of GDDY inclusion level was also noted. However, the authors recognized that factors other than mycotoxin contamination are likely to affect the upper dietary inclusion limit of yeast products as protein sources for fish. In addition, the mycotoxin contamination of the profile of the individual diets was not analyzed. Continued
efforts are therefore necessary to clearly establish the efficacy of mycotoxin-biotransforming agents in fish.

Taken together, these studies highlight the variable conclusions concerning the efficacy of mycotoxin adsorbents and biotransforming agents in different species. Multiple factors related both to the dietary treatments (e.g. type of mycotoxins, source of mycotoxins species and mycotoxin concentrations) and the animals (e.g. species, age, sex, health status and animal husbandry) can affect the observed outcomes. The endpoints chosen to assess efficacy and the experimental design are also critical. Following an in depth literature review commissioned by the EFSA, Boudergue et al. (2009) recommended that studies evaluating anti-mycotoxin feed additives should include at least the following dietary treatments: non-contaminated diet (negative control), mycotoxin-contaminated diet (positive control), non-contaminated diet with the mycotoxin-detoxifying agent and mycotoxin-contaminated diet with the mycotoxin-detoxifying agent. *In vitro* tests, although informative, may not always correlate well with *in vivo* effectiveness and should therefore not be used as the only indicator of efficacy (Diaz and Smith, 2005). These considerations are essential to ensure robust and accurate assessment of anti-mycotoxin feed additives.

2.12 – Conclusion

The risk of mycotoxin exposure inherent to the increased reliance on an expanding number of economical plant protein sources of variable origin and quality has become an emerging concern in fish nutrition (Manning, 2005; Spring and Fegan, 2005; Sissener et al., 2011; Sanden et al., 2012; Hauptman et al., 2014). The *Fusarium* mycotoxin, DON, is a feed
contaminant of predominant concern in terrestrial livestock production. Large-scale, multi-year mycotoxin surveys of feedstuffs have found DON to be a persistent problem in cereal grains and co-products of grain milling industries with the prevalence of contaminated samples consistently over 50% (Streit et al., 2013; Schatzmayr and Streit, 2013; Nährer and Kovalsky, 2014). The potential annual economic costs associated with DON contamination of feed crops and livestock losses in the U.S. alone were previously estimated to be as high as $55 million and $5 million, respectively (CAST, 2003). The most common clinical signs of chronic exposure to DON in routinely studied livestock and laboratory animals are reduced feed intake and refusal, reduced weight gain, altered nutritional efficiency and immunomodulation. The toxicity of DON is primarily related to its ability to inhibit protein synthesis in rapidly proliferating cells and tissues (Ehrlich and Daigle, 1987; Zhou et al., 2003a; Li and Pestka, 2008), regulate MAPK-dependent cellular processes including cytokine production (Moon and Pestka, 2002; Kinser et al., 2004), apoptosis (Zhou et al., 2005a,b; Bensassi et al., 2009) and mitosis (Tiemann et al., 2003; Yang et al., 2008) and alter neuroendocrine and enteroendocrine signaling within the gut-brain axis (Fitzpatrick et al., 1988; Prelusky et al., 1992; Fioramonti et al., 1993; Swamy et al., 2004; Flannery et al., 2012).

Current evidence indicates that rainbow trout are extremely sensitive to DON in feed naturally contaminated with *Fusarium* mycotoxins. Highly significant decreases in feed intake, weight gain, TGC, NRE and ERE were reported in rainbow trout fed diets containing less than 1 ppm DON (Hooft et al., 2011). In contrast, no adverse effects on feed consumption or growth were observed in juvenile channel catfish fed diets containing up to 10 ppm purified DON (Manning, 2005). Pair-feeding demonstrated that decreased performance of rainbow trout is
not exclusively related to a reduction in feed intake, but rather, associated with deleterious metabolic effects. An increasing number of studies have since investigated the effects of DON on histopathological indices, hematology, serum biochemistry, immunity, disease resistance, oxidative stress and cytotoxicity in different species and fish cell lines (Sanden et al., 2012; Manning et al., 2014; Matejova et al., 2014; Pietsch et al., 2014a,b; Pietsch and Burkhardt-Holm, 2015; Ryerse et al., 2014; 2015; Šišperová et al., 2015; Tola, 2015); however, the basis of the high sensitivity of rainbow trout to DON has not yet been elucidated. Furthermore, several fundamental questions concerning DON toxicity in fish and DON-contamination of aquaculture feeds remain to be addressed.

Systematic approaches are necessary to accurately evaluate the effects of DON on salmonids and other potentially sensitive finfish species of commercial importance. Comprehensive efforts focused on understanding the mechanisms of toxicity, identifying potential interactions between naturally co-occurring contaminants and defining species-specific responses to DON are essential in order to generate evidence-based risk assessments pertinent to fish feed manufacturers. The continually increasing relevance of mycotoxins to aquaculture nutrition also warrants the evaluation of existing mycotoxin solutions (e.g. feed additives) to minimize the negative economic impacts of mycotoxins on fish health and performance.
CHAPTER 3 – THE EFFECTS OF NATURALLY OCCURRING OR PURIFIED DEOXYNIVALENOL (DON) ON GROWTH PERFORMANCE, NUTRIENT UTILIZATION AND HISTOPATHOLOGY OF RAINBOW TROUT (ONCORHYNCHUS MYKISS)

3.1 – Abstract

It has been previously established that rainbow trout are extremely sensitive to commercially relevant levels (0 to 2 ppm) of the ubiquitous Fusarium mycotoxin deoxynivalenol (DON); however, the contribution of co-occurring mycotoxins and/or their potential interactions with DON haven not been discounted. Concurrently, there have been no systematic efforts to characterize the pathological basis of the sensitivity of rainbow trout to DON. This study was conducted to investigate these outstanding issues. Triplicate groups of rainbow trout (initial average body weight=50.3 g/fish) were fed eight diets containing graded levels of purified DON (0, 0.7, 1.4 and 2.1 ppm) or DON from naturally contaminated corn (0, 2.1, 4.1 and 5.9 ppm) for eight weeks. Fish experienced significant linear decreases in weight gain ($P<0.0001$), thermal-unit growth coefficient (TGC; $P<0.0001$) and feed intake ($P<0.0001$) regardless of the source of DON. Similarly, significant linear and/or quadratic decreases ($P<0.01$) in feed efficiency (FE), whole body crude protein (CP) content, retained nitrogen (RN), recovered energy (RE) and nitrogen retention efficiency (NRE) were observed in fish fed the diets containing either purified or naturally occurring DON. There were no significant differences ($P>0.025$) in the above mentioned parameters between fish fed the diets contaminated with the same level of purified or naturally occurring DON (2.1 ppm). In general, bi-weekly sampling (6 fish/treatment/sampling) revealed significant linear or quadratic increases in dead (apoptotic/necrotic) cells in the pyloric caeca and significant linear or quadratic decreases in mitotic cells in the pyloric caeca and liver with increasing dietary levels.
of purified DON or DON from naturally contaminated corn without any distinct histopathological abnormalities (i.e. lesions). This is in direct agreement with the known ability of DON to activate mitogen-activated protein kinases (MAPKs), suggesting that rainbow trout may be a suitable animal model for future research. Interestingly, the number of dead cells in the pyloric caeca at all sampling time points, and the degree of hepatic vacuolation after four and six weeks, were significantly different between fish fed the diet containing 2.1 ppm purified DON compared to those receiving a diet naturally contaminated with the same concentration of DON. Continued efforts are therefore needed to investigate the potential effects of mycotoxin interactions on histopathological and morphological changes of organs and tissues in more detail. However, these results demonstrate for the first time that decreased growth performance and nutrient utilization efficiency of rainbow trout exposed to grains naturally contaminated with *Fusarium* mycotoxins is most probably associated exclusively with DON.

### 3.2 – Introduction

Deoxynivalenol (DON; “vomitoxin”), a *Fusarium* mycotoxin belonging to a structurally related group of approximately 180 sesquiterpenoid compounds known as the trichothecenes, is extensively recognized as a predominant concern in human health and animal production due to its high prevalence and stability during food/feed processing (Grove, 1988, 1993, 2000; Hazel and Patel, 2004; Pestka, 2007). Recent multi-year monitoring programs have identified DON to be a persistent problem in small cereal grains (e.g. wheat, corn and barley) and co-products of grain milling industries with an incidence of contaminated samples consistently between 50% and 60% and average concentrations of up to 1.5 ppm, depending on the commodity and
region (Streit et al., 2013; Schatzmayr and Streit, 2013; Nährer and Kovalsky, 2014). The annual economic costs associated with DON contamination of food crops, feed crops and livestock losses in the United States alone have been projected to be as high as $1.2 billion, $55 million and $5 million, respectively (CAST, 2003).

The toxicity of DON varies considerably among routinely studied farm and experimental species. Pigs and rodents, notably mice, are frequently employed in mechanistic investigations of DON toxicity due, in part, to their sensitivity to DON relative to other species (i.e. poultry and ruminants). The clinical effects of high-dose, short-term exposure to DON include abdominal distress (e.g. intestinal inflammation and gastrointestinal hemorrhage), increased salivation, malaise, diarrhea and emesis (or vomiting in pigs). In comparison, common symptoms of prolonged consumption of feed contaminated with lower levels of DON include reduced weight gain, reduced feed intake (i.e. anorexia) and altered nutritional efficiency (Rotter et al., 1996; Pestka, 2007).

The cellular effects of trichothecenes are largely related to their ability to interfere with protein synthesis (Carter and Cannon, 1977; Cundliffe and Davies, 1977; Ehrlich and Daigle, 1987; Pestka, 2010a). It has been established that DON can rapidly activate mitogen-activated protein kinases (MAPKs) via the so-called ribotoxic stress response (RSR; Iordanov et al., 1997; Laskin et al., 2002). The effects of DON-mediated MAPK activation are dose- and duration-dependent: low-dose, short-term exposure produces upregulation of cytokines, chemokines and other proinflammatory-related genes and proteins resulting in immunostimulation, whereas high-dose, prolonged exposure promotes apoptosis resulting in tissue damage and immunosuppression (Li et al., 1997; Moon and Pestka, 2002; Chung et al., 2003a,b; Tiemann et
The main pathological consequences of high-dose exposure to DON in experimental animals include marked necrosis of the gastrointestinal tract and lymphoid tissues (Arnold et al., 1986; Forsell et al., 1987; Robbana-Barnat et al., 1987). In contrast, the adverse health effects of chronic consumption of DON at levels corresponding to the majority of advisory or regulatory maxima for human food (0.2-2.0 ppm) are not yet clearly defined (FAO, 2004; Pestka and Smolinski, 2005).

Recent evidence suggests that rainbow trout are extremely sensitive to commercially relevant levels of DON (< 2 ppm). This species may therefore represent a useful animal model in continued efforts to better understand the risks associated with low-dose exposure to DON (Hooft et al., 2011). Highly significant decreases in weight gain, growth rate (thermal-unit growth coefficient, TGC), feed intake, feed efficiency (FE), whole body crude protein (CP) content, nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were observed in juvenile rainbow trout fed diets containing 0.3, 0.8, 1.4, 2.0 and 2.6 ppm DON from naturally contaminated corn for eight weeks (Hooft et al., 2011). The overt symptoms of chronic exposure to DON in rainbow trout (reduced weight gain, feed intake and FE) were in good agreement with those previously reported with growing pigs fed diets containing similar levels (1-2 ppm) of DON (Young et al., 1993; Smith et al., 1997). Nonetheless, when DON intake of starter pigs (data from Smith et al., 1997) and rainbow trout were adjusted according to average metabolic body weight, daily weight gain of rainbow trout appeared to be more severely affected by DON (Hooft et al., 2011). Pair-feeding confirmed that decreased
performance and nutrient retention were most likely due to deleterious metabolic effects and not exclusively related to decreased feed intake. This contention was supported by preliminary, but unsystematic, gross and histological assessment of selected tissues (liver and intestine) which revealed subcapsular hemorrhage and morphological changes of the livers of some fish fed diets containing 1.4, 2.0 and 2.6 ppm DON (Hooft et al., 2011). However, the basis of the sensitivity of rainbow trout to DON has not been elucidated. Likewise, the possible contribution of co-occurring mycotoxins present in the naturally contaminated corn to the detrimental effects of the experimental diets on fish health and performance could not be discounted. Indeed, the onset of DON-like symptoms in commercial swine operations despite negligible concentrations of DON in the feed has been previously attributed to the presence of additional mycotoxins and/or their toxicological interactions with DON (Trenholm et al., 1983; Abbas et al., 1986; Smith et al., 1997; CAST, 2003).

Based on these unresolved questions, the purpose of this study was two-fold: first, to determine if reduced growth performance of rainbow trout fed diets naturally contaminated with *Fusarium* mycotoxins is primarily associated with DON or, whether it is the result of interactive effects related to the presence of multiple mycotoxins; and secondly, to thoroughly and systematically evaluate the effects of DON on histopathological changes of organs and tissues in rainbow trout. These objectives represent an initial step in the validation of rainbow trout as an alternative, potentially valuable experimental animal model in support of efforts focused on expanding our knowledge of the chronic effects of feed-borne DON at levels relevant to human and animal health.
3.3 – Materials and methods

3.3.1 – Fish husbandry and experimental conditions

Rainbow trout obtained from the Alma Aquaculture Research Station (Elora, ON, Canada) were acclimated to the experimental conditions for two weeks prior to the start of the trial. During this time, fish were fed a maintenance ration of a high quality commercial trout feed (Martin Mills Inc., Elmira, Ontario, Canada) once daily.

Groups of 18 fish with an initial average body weight of 50.3 g/fish were randomly assigned to 24 tanks, with three replicate tanks per diet. Fish were maintained in a flow-through system comprised of 60 L fibreglass tanks, individually aerated and supplied with well-water at a rate of 3 L/min. Water temperature was maintained at 12.4 ± 0.5 °C and a photoperiod of 12 hours light: 12 hours dark was employed. The animals were kept in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984) and the University of Guelph Animal Care Committee.

Over the eight-week experimental period, feed intake was recorded weekly and individual tanks of fish were weighed every 28 days. Prior to the start of the experiment, a pooled sample of ten fish was taken for determination of initial carcass composition. Similarly, at the end of the trial, four fish per tank were randomly sampled for determination of final carcass composition. An initial pooled sample of six fish was taken to confirm the absence of any relevant background histopathological lesions. Subsequently, two fish per tank were randomly sampled for histopathological analysis every two weeks (at weeks two, four, six and eight). Fish were fasted for 24 hours prior to sampling for histopathological analysis. Fish were humanely euthanized by an overdose of tricaine methane sulfonate (200 mg/L water).
3.3.2 – Experimental diets and feeding protocol

In total, eight experimental diets were formulated to meet the digestible energy (DE), digestible protein (DP), amino acid, vitamin and mineral requirements for rainbow trout according to NRC (2011). Two sets of four diets each, one containing graded levels of purified DON and one containing graded levels of DON from naturally contaminated corn, were manufactured (Table 3.1). Diets 1 (control), 2, 3 and 4 were formulated to contain purified DON with targeted concentrations of 0, 1, 2 and 3 ppm, respectively. The levels of purified DON in Diets 2, 3 and 4 were achieved by inclusion of contaminated mineral premix. Briefly, vials of purified DON (Sigma-Aldrich, Oakville, ON, Canada) were dissolved in ethanol and added to a pre-determined quantity (90 g) of mineral premix. Following mixing using a bench-scale mixer (DuPont Instruments, Newtown, CT, USA), the ethanol was allowed to evaporate overnight under a fume hood. The contaminated mineral premix was re-ground and combined with clean (uncontaminated) mineral premix in a ratio that was calculated to give the desired dietary concentration of DON based on an inclusion of 1 g mineral premix/100 g diet.

Diets 5 (control), 6, 7 and 8 were formulated to contain DON from naturally contaminated corn (Table 3.1). Two batches of ground corn (< 2 mm) were obtained from the Arkell Feed Mill (University of Guelph, Guelph, ON, Canada). Graded levels of DON in Diets 5 (control), 6, 7 and 8 were achieved by incremental replacement of “clean” corn with contaminated corn as previously described by Hooft et al. (2011). The desired concentrations of DON in these naturally contaminated diets were the same as those targeted in the diets containing purified DON (0, 1, 2 and 3 ppm). Inclusion of corn protein concentrate (CPC) and
raw corn starch were adjusted to balance the contribution of corn and corn-based ingredients to total dietary crude protein and starch content.

Diets were mixed using a Hobart mixer (Hobart Ltd., Don Mills, ON, Canada), steam pelleted using a laboratory pellet mill (California Pellet Mill Co., San Francisco, CA, USA), dried under forced-air at room temperature for 24 hours, sieved and stored at 4 °C until used. Fish were hand-fed to satiety three times daily on weekdays and once per day on weekends.

### 3.3.3 – Mycotoxin analysis

The formulation of the naturally contaminated diets (i.e. the inclusion levels of contaminated corn needed to achieve the desired concentrations of DON) was based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the corn sources conducted prior to the study (Malachová et al., 2014; Department for Agrobiotechnology, IFA-Tulln, University of Natural Resources and Applied Life Sciences, Tulln, Austria). Following diet preparation, representative samples of the two batches of corn and the eight experimental diets were analyzed for DON, 3-acetyl DON (3-ADON), 15-acetyl DON (15-ADON), nivalenol (NIV), T-2 toxin, iso T-2 toxin, acetyl-T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, fusarenone-X, diacetoxyscirpenol (DAS), scirpentriol, 15-acetoxyscirpentriol, neosolaniol, zearalenone (ZON) and zearalenol by gas chromatography (GC)/mass spectrometry (MS). Aflatoxin B₁ (AFB₁) and fumonisin B₁ (FB₁) were analyzed by high performance liquid chromatography (HPLC) and fluorescence detection (Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, ND, USA). The practical quantitation limit (PQL) for all mycotoxins was 0.5 ppm, with the exception of AFB₁ (0.02 ppm) and FB₁ (2 ppm).
Table 3.1. Formulation of the experimental diets and dietary mycotoxin concentrations.

<table>
<thead>
<tr>
<th>Ingredient (g/100 g diet)</th>
<th>Diet</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal, herring</td>
<td></td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Poultry by-product meal</td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Corn, clean&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.0</td>
<td>18.0</td>
<td>11.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Corn, contaminated&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.0</td>
<td>14.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Corn protein concentrate</td>
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<td>7.6</td>
<td>7.6</td>
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<td>17.48</td>
<td>-</td>
<td>1.2</td>
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<td>Wheat gluten</td>
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<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
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<tr>
<td>Biolys&lt;sup&gt;c&lt;/sup&gt;, 52 % lysine</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Monocalcium phosphate</td>
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<td>1.0</td>
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<td>1.0</td>
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<td>1.0</td>
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<tr>
<td>Vitamin premix&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1.0</td>
<td>1.0</td>
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<tr>
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<td>1.0</td>
<td>1.0</td>
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<td>Cellulose (Arbocel)</td>
<td></td>
<td>6.62</td>
<td>6.6199</td>
<td>6.6198</td>
<td>6.6197</td>
<td>2.79</td>
<td>1.86</td>
<td>0.92</td>
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<tr>
<td>Purified deoxynivalenol</td>
<td></td>
<td>-</td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.0003</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Fish oil, herring</td>
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<td>11.0</td>
<td>11.0</td>
<td>11.0</td>
<td>11.0</td>
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<td>5.2</td>
<td>4.6</td>
<td>4.6</td>
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<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>100</td>
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Analyzed composition (dry matter basis)

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<tr>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>94.2</td>
<td>93.5</td>
<td>93.9</td>
<td>94.1</td>
<td>94.5</td>
<td>94.2</td>
<td>94.5</td>
<td>95.1</td>
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<tr>
<td>Crude protein (%)</td>
<td>41.5</td>
<td>41.6</td>
<td>41.3</td>
<td>40.2</td>
<td>43.4</td>
<td>41.7</td>
<td>43.3</td>
<td>40.9</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>20.7</td>
<td>20.7</td>
<td>21.1</td>
<td>21.0</td>
<td>20.9</td>
<td>20.8</td>
<td>20.6</td>
<td>21.1</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.5</td>
<td>8.6</td>
<td>9.0</td>
<td>8.5</td>
<td>9.7</td>
<td>9.2</td>
<td>10.8</td>
<td>9.3</td>
</tr>
<tr>
<td>Gross energy (KJ/g)</td>
<td>23.4</td>
<td>23.2</td>
<td>23.2</td>
<td>23.4</td>
<td>23.6</td>
<td>23.3</td>
<td>23.1</td>
<td>23.3</td>
</tr>
</tbody>
</table>

Analyzed mycotoxin concentrations (ppm)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxynivalenol (DON)</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.7</td>
<td>1.4</td>
<td>2.1</td>
<td>ND</td>
<td>2.1</td>
<td>4.1</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Zearalenone (ZON)</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.5</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Contains: 0.9 ppm deoxynivalenol (DON).
<sup>b</sup>Contains: 26.8 ppm DON, 1.4 ppm 15-acetyl DON and 2.2 ppm zearalenone (ZON).
<sup>c</sup>HP 300 (Hamlet Protein, Horsens Denmark).
<sup>d</sup>Provides per kg of diet: retinyl acetate (vitamin A), 75 mg; cholecalciferol (vitamin D3), 300 mg; menadione Na-bisulfate (vitamin K), 1.5 mg; cyanocobalamine (vitamin B12), 30 mg; D-biotin, 210 mg; choline chloride, 3448 mg; folic acid, 1.5 mg; niacin, 15 mg; calcium-d-pantothenate, 33 mg; pyridoxine–HCl, 7.5 mg; riboflavin, 9 mg; thiamin–HCl, 1.5 mg (Martin Mills Inc., Elmira, ON, Canada).
<sup>e</sup>Provides per kg of diet: sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; potassium iodine (KI, 24% K, 76% I), 10.5 mg; ferrous sulphate (FeSO<sub>4</sub>, 7H<sub>2</sub>O, 20% Fe), 65 mg; manganese sulphate (MnSO<sub>4</sub>, 36% Mn) 88.9 mg; zinc sulphate (ZnSO<sub>4</sub>, 7H<sub>2</sub>O, 40% Zn), 150 mg; copper sulphate (CuSO<sub>4</sub>, 5H<sub>2</sub>O, 25% Cu), 28 mg; sodium Selenite (Na<sub>2</sub>SeO<sub>3</sub>, 45.66% Se), 0.7 mg (Martin Mills Inc., Elmira, ON, Canada).
<sup>f</sup>ND=not detectable.
3.3.4 – Chemical analysis

Diets, ingredients and carcass samples were analyzed for dry matter (DM) and ash according to AOAC (1995), crude protein (CP, %N × 6.25) by LECO (LECO Corp., St. Joseph, MI, USA), lipids with an Ankom XT20 fat analyzer (Ankom Technology, Macedon, NY, USA) using petroleum ether and gross energy (GE) content using a Parr 1271 automated bomb calorimeter (Parr Instruments, Moline, IL, USA). Prior to analysis, diet and ingredient samples were ground to a fine, homogenous consistency. Fish analyzed for carcass composition were cooked in an autoclave, ground into a homogenous slurry using a food processor, freeze dried, reground and stored at –20 °C until proximate analysis.

3.3.5 – Histopathological analysis

Fish were fixed in 10% buffered formalin using a formalin to tissue ratio of 10:1 for 24 to 48 hours following removal of one side of the body wall (via an abdominal incision along the ventral midline), the opercula and the tail. Small incisions were made in the stomach and at the junction of the small intestine and large intestine. The intestine was detached at the vent and the internal organs were carefully pulled away from the body cavity to allow for proper fixation. Sections of tissues and organs including the stomach, pyloric caeca, small intestine, large intestine, liver, spleen, heart, anterior kidney, posterior kidney, swim bladder, gill arches, eye, brain and muscle were excised, placed into cassettes and decalcified. Samples were dehydrated, embedded in paraffin, sectioned at 5 µm, mounted on glass slides and stained with H&E by the Animal Health Laboratory (University of Guelph, Guelph, ON, Canada).
Based on preliminary scans of selected organs and tissues from fish fed the control diets (Diets 1 and 5) and fish fed the most highly contaminated diets (Diets 4 and 8) at each sampling interval, parameters of potential interest were established: the number of dead (apoptotic and necrotic) cells and mitotic figures in the pyloric caeca, the number of mitotic cells in the liver and the degree of vacuolation of the liver. Dead and mitotic cells in three folds per pyloric caeca from three different pyloric caeca (i.e. 9 folds per fish) were counted where possible (Figure 3.1). Similarly, the number of mitotic cells was counted in ten fields per liver. Vacuolation of the liver was assessed according to a scoring system developed based on the preliminary scans: (1) absent to slight or focal alteration; (2) moderate; (3) severe or present throughout the tissue (Figure 3.2). All analyses were performed using bright field light microscopy on a Leica DMR light microscope (Leica Microsystems Inc., Concord, ON, Canada) equipped with Openlab imaging software (Openlab 5.5, PerkinElmer, Waltham, MA, USA).
Figure 3.1. Pyloric caeca of rainbow trout showing dead cells (arrows) and a mitotic figure (arrow head) (H&E, ×400).

Figure 3.2. Vacuolation of the liver of rainbow trout showing (1) absent to slight or focal alteration; (2) moderate vacuolation; and (3) severe vacuolation (present throughout the tissue) (H&E, ×400).
3.3.6 – Calculations

Growth rate, expressed as thermal-unit growth coefficient (TGC), was calculated for each tank as: \[ TGC = 100 \times \left[ \left( FBW^{1/3} - IBW^{1/3} \right) \times \left( \text{sum } T \times D \right)^{-1} \right] \], where: FBW = final body weight (g/fish); IBW = initial body weight (g/fish); and sum T × D = sum degrees Celsius × days.

Feed efficiency (FE, gain:feed) was calculated for each tank as: \[ FE = \frac{\text{live body weight gain}}{\text{dry feed intake}} \], where: feed intake = total dry feed/number of fish; and live body weight gain = (FBW/final number of fish) – (IBW/initial number of fish).

Hepatosomatic index (HSI) was calculated for each fish as: \[ HSI = \frac{[\text{final liver weight} (g) / \text{FBW}]}{100}. \]

Retained nitrogen (RN, g/fish) and recovered energy (RE, KJ/fish) were calculated for each tank as: \[ RN = (FBW \times \text{N content}_{\text{final}}) - (IBW \times \text{N content}_{\text{initial}}) \] and \[ RE = (FBW \times \text{GE content}_{\text{final}}) - (IBW \times \text{GE content}_{\text{final}}) \], respectively, where: N content_{final} = nitrogen content (%) of the final carcass sample; N content_{initial} = nitrogen content (%) of the initial carcass sample; GE_{final} = gross energy (KJ/g) content of the final carcass sample; and GE_{initial} = gross energy (KJ/g) content of the initial carcass sample.

Nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were calculated for each tank as a percentage of ingested nitrogen (IN, g/fish) and ingested energy (IE, KJ/fish), respectively: \[ \text{NRE} \% \text{ IN} = \frac{\left( (FBW \times \text{N content}_{\text{final}}) - (IBW \times \text{N content}_{\text{initial}}) \right)}{\text{IN}} \times 100 \] and \[ \text{ERE} \% \text{ IE} = \frac{\left( (FBW \times \text{GE content}_{\text{final}}) - (IBW \times \text{GE content}_{\text{initial}}) \right)}{\text{IE}} \times 100. \]
### 3.3.7 Statistical Analysis

All growth performance, chemical body composition and nutrient utilization data were analyzed as a randomized incomplete block design using the PROC MIXED procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). Block was included in the model as a random effect and tank was considered the experimental unit. Residual plots were examined and the Brown and Forsythe test was used to ensure homogeneity of variances prior to any other statistical analysis. Linear and quadratic orthogonal polynomial contrasts across graded levels of DON within each source were used to evaluate the responses for all dependent variables. Contrast coefficients for the measured, graded levels of DON were generated using the PROC IML procedure of SAS. An independent, non-orthogonal contrast was used to compare the response of fish fed Diet 4 and those fed Diet 6 for the dependent variables.

Histopathological data were analyzed using repeated measures in the PROC MIXED procedure of SAS. Transformation of dependent variables was adopted as necessary: log (dependent variable+1) for the number of mitotic (in pyloric caeca and liver) and dead (in pyloric caeca) cells and log (score) for vacuolation score (of the liver). Block was included in the model as a random effect and the best-fitting covariance structure for each dependent variable was determined according to the Akaike criterion (Kuehl, 2000). Linear and quadratic polynomial contrasts across graded levels of DON within each source of DON were used to evaluate the responses for all dependent variables at each sampling time. An independent, non-orthogonal contrast was used to compare the response of fish fed Diet 4 and those fed Diet 6 for the dependent variables at each sampling time. A Bonferroni correction was applied
and statistical significance was declared at $P \leq 0.025$ for all dependent variables. Trends were noted at $P \leq 0.05$.

3.4 – Results

3.4.1 – Mycotoxin concentrations

The results of the LC-MS/MS analysis of the two batches of corn available prior to the study indicated concentrations of 0.4 and 14.6 ppm DON in the clean and contaminated sources, respectively. However, these findings were different than those obtained by GC-MS following the formulation of the experimental diets. The clean corn was found to contain 0.9 ppm DON, while the contaminated source contained 26.8 ppm DON. A complete comparison of the mycotoxin analysis of the two corn sources conducted by LC-MS/MS and GC-MS is presented in Table 3.2. Due to the discrepancies in the DON content of the contaminated corn determined by LC-MS/MS and HPLC-MS, analysis of the naturally contaminated diets conducted using HPLC-MS indicated higher levels of DON than targeted and the interaction of dietary DON concentration and source (purified vs. naturally contaminated) could not be statistically evaluated. Diet 1 (control, purified) and Diet 5 (control, naturally contaminated) did not contain detectable levels of any of the analyzed mycotoxins. Diets 2, 3 and 4, formulated using purified DON, were found to contain 0.7, 1.4 and 2.1 ppm DON, respectively. Diets 6, 7 and 8, prepared by increasing inclusion of contaminated corn, contained 2.1, 4.1 and 5.9 ppm DON, respectively (Table 3.1). Additionally, ZON was present in Diets 6 and 7 (0.5 and 0.6 ppm, respectively).
3.4.2 – Growth performance

The growth curves of fish fed the experimental diets are presented in Figure 3.3. Significant linear decreases in weight gain, TGC, FI (P<0.0001) and FE (P<0.01) of fish fed diets with increasing concentrations of purified DON were observed (Table 3.3). Similarly, significant linear decreases in weight gain and TGC (P<0.0001) were associated with feeding diets containing graded levels of DON from naturally contaminated corn (Figure 3.4). FI decreased linearly (P<0.0001) in response to increasing levels of DON in the naturally contaminated diets, while significant linear (P<0.0001) and quadratic (P<0.001) decreases in FE of fish fed these diets were observed. Hepatosomatic index (HSI) was not affected by increasing levels of purified DON. Conversely, feeding naturally contaminated diets resulted in a significant linear decrease (P<0.025) of this parameter. No significant differences in any growth performance parameters were observed between fish fed Diet 4 (2.1 ppm purified DON) and fish fed Diet 6 (2.1 ppm DON from naturally contaminated corn; Table 3.3).
Table 3.2. Comparison of the mycotoxin analysis of the contaminated corn by LC-MS/MS and GC-MS.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LC-MS/MS (ppm)</th>
<th>GC-MS (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trichothecenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>14.56</td>
<td>26.8</td>
</tr>
<tr>
<td>DON-3-glucoside</td>
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<tr>
<td>3-ADON</td>
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<td>ND</td>
</tr>
<tr>
<td>15-ADON</td>
<td>N/A</td>
<td>1.4</td>
</tr>
<tr>
<td>NIV</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.01</td>
<td>ND</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>0.05</td>
<td>ND</td>
</tr>
<tr>
<td><strong>ZON and its derivatives or conjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZON</td>
<td>1.26</td>
<td>2.2</td>
</tr>
<tr>
<td>α-zearalenol</td>
<td>0.04</td>
<td>N/A</td>
</tr>
<tr>
<td>β-zearalenol</td>
<td>0.03</td>
<td>N/A</td>
</tr>
<tr>
<td>ZON glucoside</td>
<td>0.01</td>
<td>N/A</td>
</tr>
<tr>
<td>α-zearalenol glucoside</td>
<td>0.11</td>
<td>N/A</td>
</tr>
<tr>
<td>β-zearalenol glucoside</td>
<td>0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>ZON 4-sulfate</td>
<td>0.05</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Other Fusarium metabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culmorin</td>
<td>0.09</td>
<td>N/A</td>
</tr>
<tr>
<td>5-hydroxyculmorin</td>
<td>0.47</td>
<td>N/A</td>
</tr>
<tr>
<td>Aurofusarin</td>
<td>9.84</td>
<td>N/A</td>
</tr>
<tr>
<td>Fusaric acid</td>
<td>0.05</td>
<td>N/A</td>
</tr>
<tr>
<td>Cyclosporin C</td>
<td>0.02</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Aspergillus metabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloaspeptide A</td>
<td>0.16</td>
<td>N/A</td>
</tr>
<tr>
<td>Paspalin</td>
<td>1.61</td>
<td>N/A</td>
</tr>
<tr>
<td>Terphenyllin</td>
<td>0.04</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Other fungal metabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brevainamide F</td>
<td>0.03</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Metabolites analyzed, but not detected by GC-MS: iso T-2 toxin, acetyl-T-2-toxin, T-2 triol, T-2 tetraol, fusarenone-X, DAS, scirpentriol, 15-acetoxyscirpentriol, neosolaniol, zearalenol, FB1, AFB1. FB1 and AFB1 were analyzed by HPLC and fluorescence detection.

Metabolites analyzed, but not detected by LC-MS/MS: neosolaniol, butenolid, equisetin, cyclosporin D, cyclosporin H, asperloxin A, cyclopeptine, radicicol.

Metabolites analyzed by LC-MS/MS, but present at concentrations < 0.01 ppm: beauvericin, enniatin B, enniatin B1, apicidin, cyclosporin A, sterigmatocystin, emodin.

ND=not detectable. See Malachová et al. (2014) for LOD and LOQ for each metabolite analyzed by LC-MS/MS. The PQL for the GC-MS analysis was 0.5 ppm for all mycotoxins, with the exception of 0.02 ppm for AFB1 and 2 ppm for FB1.

N/A=not applicable (metabolite not analyzed).

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Table 3.3. Weight gain, thermal-unit growth coefficient, feed intake and feed efficiency of rainbow trout (initial average weight=50.3 g/fish) fed the experimental diets for 56 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gain (g/fish)</th>
<th>TGC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Feed intake (g/fish)</th>
<th>FE&lt;sup&gt;c&lt;/sup&gt; (gain/feed)</th>
<th>HSI&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>P</td>
<td>144.0</td>
<td>0.303</td>
<td>137.8</td>
<td>1.06</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>P</td>
<td>132.4</td>
<td>0.287</td>
<td>127.1</td>
<td>1.03</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>P</td>
<td>98.0</td>
<td>0.230</td>
<td>105.6</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>P</td>
<td>88.4</td>
<td>0.215</td>
<td>91.1</td>
<td>0.98</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Significance**

**Linear**
- P<0.0001
- P<0.0001
- P<0.0001
- P<0.01
- N.S.

**Quadratic**
- N.S.
- N.S.
- N.S.
- N.S.
- N.S.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gain (g/fish)</th>
<th>TGC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Feed intake (g/fish)</th>
<th>FE&lt;sup&gt;c&lt;/sup&gt; (gain/feed)</th>
<th>HSI&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0</td>
<td>C</td>
<td>140.8</td>
<td>0.299</td>
<td>132.5</td>
<td>1.06</td>
<td>1.07</td>
</tr>
<tr>
<td>6</td>
<td>2.1</td>
<td>C</td>
<td>83.5</td>
<td>0.205</td>
<td>85.2</td>
<td>1.00</td>
<td>1.07</td>
</tr>
<tr>
<td>7</td>
<td>4.1</td>
<td>C</td>
<td>55.3</td>
<td>0.150</td>
<td>52.6</td>
<td>1.05</td>
<td>1.10</td>
</tr>
<tr>
<td>8</td>
<td>5.9</td>
<td>C</td>
<td>34.0</td>
<td>0.099</td>
<td>40.7</td>
<td>0.80</td>
<td>0.79</td>
</tr>
</tbody>
</table>

**Significance**

**Linear**
- P<0.0001
- P<0.0001
- P<0.0001
- P<0.0001
- P=0.0149

**Quadratic**
- N.S.
- N.S.
- P=0.0232
- P<0.001
- P=0.0238

**Diet 4 vs. Diet 6**
- N.S.
- N.S.
- N.S.
- N.S.
- N.S.

**S.E.M.**
- 6.7
- 0.011
- 6.0
- 0.02
- 0.06

---

<sup>a</sup>Source of DON; P=purified DON, C=naturally contaminated corn.
<sup>b</sup>TGC=thermal-unit growth coefficient
<sup>c</sup>FE=feed efficiency
<sup>d</sup>HSI=Hepatosomatic index
<sup>e</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of purified DON.
<sup>f</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON from naturally contaminated corn.
<sup>g</sup>Significance of the non-orthogonal contrast of dependent variables for Diet 4 vs. Diet 6.
<sup>h</sup>S.E.M.=standard error mean.
<sup>i</sup>N.S.=not statistically significant
Figure 3.3. Growth curves of rainbow trout fed the experimental diets containing graded levels of DON from a purified source (A) or naturally contaminated corn (B).
Figure 3.4. Growth rate (TGC) of rainbow trout (IBW=50.3 g/fish) fed the experimental diets containing purified DON or naturally contaminated corn for 56 days.
Figure 3.5. Carcass crude protein (CP, %) content of rainbow trout (IBW=50.3 g/fish) fed the experimental diets containing purified DON or naturally contaminated corn for 56 days.
3.4.3 – Carcass composition and nutrient utilization

Feeding diets containing increasing levels of purified DON resulted in a significant linear (P<0.01) decrease in whole body CP content. Significant linear and quadratic (P<0.001) decreases in CP content of fish fed the naturally contaminated diets were also observed (Table 3.4; Figure 3.5). Significant decreases in whole body contents of water, lipid (P<0.0001) and GE (P<0.001) were associated with feeding diets containing increasing levels of DON from naturally contaminated corn; however, these differences were not reflected in the carcass composition of fish fed the diets contaminated with purified DON (Table 3.4).

RN (P<0.0001), RE (P<0.001) and NRE (P<0.01) decreased linearly as the dietary concentration of purified DON increased. Likewise, significant linear (P<0.0001) and quadratic (P<0.01) decreases in RN and significant linear decreases in RE, NRE and ERE (P<0.0001) were found in fish fed diets containing increasing levels of naturally occurring DON. In accordance with the growth performance results, carcass composition and nitrogen and energy utilization efficiencies did not differ significantly between fish fed Diet 4 and those fed Diet 6 (Table 3.5).
Table 3.4. Chemical body composition of the whole carcass of rainbow trout (initial average weight=50.3 g/fish) fed the experimental diets for 56 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>Source(^a)</th>
<th>H(_2)O (%)</th>
<th>CP(^b) (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>GE(^c) (KJ/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>P</td>
<td>69.6</td>
<td>16.7</td>
<td>11.6</td>
<td>2.9</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>P</td>
<td>71.0</td>
<td>16.3</td>
<td>10.5</td>
<td>2.8</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>P</td>
<td>70.0</td>
<td>16.1</td>
<td>12.0</td>
<td>2.3</td>
<td>8.5</td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>P</td>
<td>70.1</td>
<td>16.0</td>
<td>11.9</td>
<td>2.5</td>
<td>8.4</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>C</td>
<td>69.7</td>
<td>16.9</td>
<td>11.3</td>
<td>2.8</td>
<td>8.5</td>
</tr>
<tr>
<td>6</td>
<td>2.1</td>
<td>C</td>
<td>70.6</td>
<td>16.0</td>
<td>11.2</td>
<td>3.1</td>
<td>8.0</td>
</tr>
<tr>
<td>7</td>
<td>4.1</td>
<td>C</td>
<td>73.1</td>
<td>15.7</td>
<td>8.4</td>
<td>3.2</td>
<td>7.2</td>
</tr>
<tr>
<td>8</td>
<td>5.9</td>
<td>C</td>
<td>74.9</td>
<td>16.1</td>
<td>6.4</td>
<td>3.6</td>
<td>6.7</td>
</tr>
</tbody>
</table>

\(^d\)Significance

**Linear**
- N.S.\(^h\)
- P<0.01
- N.S.
- N.S.
- N.S.

**Quadratic**
- N.S.
- N.S.
- N.S.
- N.S.
- N.S.

**Significance\(^e\)**

**Linear**
- P<0.0001
- P<0.001
- P<0.0001
- N.S.
- P<0.001

**Quadratic**
- N.S.
- P<0.001
- N.S.
- N.S.
- N.S.

**Diet 4 vs. Diet 6\(^f\)**
- N.S.
- N.S.
- N.S.
- N.S.
- N.S.

**S.E.M.\(^g\)**
- 0.7
- 0.1
- 0.6
- 0.3
- 0.3

\(^a\)Source of DON; P=purified DON, C=naturally contaminated corn.
\(^b\)CP=crude protein.
\(^c\)GE=gross energy.
\(^d\)Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of purified DON.
\(^e\)Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON from naturally contaminated corn.
\(^f\)Significance of the non-orthogonal contrast of dependent variables for Diet 4 vs. Diet 6.
\(^g\)S.E.M.=standard error mean.
\(^h\)N.S.=not statistically significant.
Table 3.5. Retained nitrogen, recovered energy, nitrogen retention efficiency and energy retention efficiency of rainbow trout (initial average weight=50.3 g/fish) fed the experimental diets for 56 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RN&lt;sup&gt;b&lt;/sup&gt; (g/fish)</th>
<th>RE&lt;sup&gt;c&lt;/sup&gt; (KJ/fish)</th>
<th>NRE&lt;sup&gt;d&lt;/sup&gt; (% IN)</th>
<th>ERE&lt;sup&gt;e&lt;/sup&gt; (% IE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>P</td>
<td>3.9</td>
<td>1352</td>
<td>43.6</td>
<td>41.6</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>P</td>
<td>3.6</td>
<td>1132</td>
<td>41.5</td>
<td>38.1</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>P</td>
<td>2.6</td>
<td>969</td>
<td>37.4</td>
<td>39.0</td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>P</td>
<td>2.3</td>
<td>863</td>
<td>39.7</td>
<td>40.4</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>C</td>
<td>4.0</td>
<td>1309</td>
<td>43.0</td>
<td>42.5</td>
</tr>
<tr>
<td>6</td>
<td>2.1</td>
<td>C</td>
<td>2.2</td>
<td>780</td>
<td>39.6</td>
<td>39.2</td>
</tr>
<tr>
<td>7</td>
<td>4.1</td>
<td>C</td>
<td>1.5</td>
<td>440</td>
<td>39.7</td>
<td>36.7</td>
</tr>
<tr>
<td>8</td>
<td>5.9</td>
<td>C</td>
<td>1.0</td>
<td>237</td>
<td>34.6</td>
<td>24.7</td>
</tr>
</tbody>
</table>

**Significance<sup>f</sup>**

<table>
<thead>
<tr>
<th>Linear</th>
<th>Quadratic</th>
<th>Diet 4 vs. Diet 6&lt;sup&gt;h&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;0.0001</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>P&lt;0.01</td>
<td>P=0.0270</td>
<td>N.S.</td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>S.E.M.&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>82</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Source of DON; P=purified DON, C=naturally contaminated corn.

<sup>b</sup>RN=retained nitrogen.

<sup>c</sup>RE=recovered energy.

<sup>d</sup>NRE (% IN)=nitrogen retention efficiency (% ingested nitrogen).

<sup>e</sup>ERE (% IE)=energy retention efficiency (% ingested energy).

<sup>f</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of purified DON.

<sup>g</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON from naturally contaminated corn.

<sup>h</sup>Significance of the non-orthogonal contrast of dependent variables for Diet 4 vs. Diet 6.

<sup>i</sup>S.E.M.=standard error mean.

<sup>j</sup>N.S.=not statistically significant.
3.4.4 – Histopathological effects

No distinct histopathological abnormalities (i.e. lesions) were noted in any tissues or organs of fish fed the experimental diets. Systematic analysis of the pyloric caeca indicated significant effects of diet ($P \leq 0.0001$) and time ($P \leq 0.01$) on the number of dead cells per fold, but no significant interaction of these variables. Interestingly, significant linear and/or quadratic increases in the number of dead cells per fold occurred in response to feeding diets containing increasing concentrations of purified DON at each sampling interval (Figure 3.6). Similarly, significant quadratic increases in the number of dead cells per fold were associated with increasing levels of DON from contaminated corn after 4 ($P \leq 0.01$) and 8 ($P \leq 0.001$) weeks (Figure 3.6).

The prevalence of mitotic cells in the pyloric caeca was significantly affected by diet ($P \leq 0.001$) and time ($P \leq 0.0001$). The number of mitotic cells per fold decreased linearly ($P \leq 0.01$) with increasing levels of DON from naturally contaminated corn after 8 weeks; linear trends toward decreases were also apparent at week 2 ($P=0.0316$) and week 4 ($P=0.0367$). Feeding diets containing purified DON resulted in a quadratic effect on the number of mitotic cells per fold after 2 weeks ($P \leq 0.025$) and a quadratic decrease after 4 weeks ($P \leq 0.025$) (Figure 3.7). The number of dead cells per fold in the pyloric caeca was significantly higher in fish fed Diet 4 compared to those fed Diet 6 at each sampling time point (Figure 3.6). In contrast, no significant differences in the number of mitotic cells per fold between fish fed Diet 4 and Diet 6 were observed (Figure 3.7).

Initial analysis of liver sections from fish fed the control and most highly contaminated diets at each time indicated a low prevalence of dead cells (i.e. 0-1 per field). Consequently,
only the mitotic cells were counted in this tissue. The number of mitotic cells per field of liver was significantly affected by diet ($P \leq 0.0001$) and sampling time ($P \leq 0.0001$). The interaction (diet $\times$ time) was also significant ($P \leq 0.001$), suggesting that the effect of diet on the number of mitotic cells per field of liver varied with time. The number of mitotic cells per field decreased linearly with increasing levels of purified DON after 2 ($P \leq 0.025$) and 6 ($P \leq 0.01$) weeks and quadratically after 4 weeks ($P \leq 0.025$). Feeding diets containing increasing, graded levels of naturally occurring DON resulted in a significant linear increase ($P \leq 0.01$) in the number of mitotic cells per field at week 2, but a trend toward a linear decrease ($P = 0.0301$) after 6 weeks. As in the pyloric caeca, there were no significant differences in the number of mitotic cells per field in fish fed Diet 4 compared to those fed Diet 6 (Figure 3.8).

Vacuolation score of the liver was significantly affected by both diet ($P \leq 0.0001$) and time ($P \leq 0.01$) and a significant interaction between diet and time ($P \leq 0.01$) was noted. However, no significant linear or quadratic effects in the vacuolation score across graded levels of DON from a purified or naturally contaminated source were found. There was a trend ($P = 0.0390$) toward increased vacuolation after 8 weeks of feeding diets containing increasing concentrations of DON from naturally contaminated corn. The vacuolation score of livers from fish fed Diet 6 was significantly higher ($P \leq 0.01$) than that of fish fed Diet 4 after 4 and 6 weeks of the experimental period (Figure 3.9).
Figure 3.6. Dead (apoptotic and necrotic) cells in the pyloric caeca of rainbow trout fed the experimental diets for 56 days. Values are mean number of dead cells/fold + SEM (6 fish/diet/time; 9 folds/fish where possible). Significance of the linear (L) and quadratic (Q) orthogonal polynomial contrasts of the dependent variable across graded levels of DON from a purified (P) or naturally contaminated (C) source at each sampling time are indicated (* P≤0.025; ** P≤0.01; *** P≤0.001; **** P≤0.0001). Significance of the non-orthogonal contrast of Diet 4 (purified, 2.1 ppm DON) vs. Diet 6 (naturally contaminated, 2.1 ppm DON) at each sampling time is indicated († P≤0.025; # P≤0.01; ¥ P=0.0001). Diet: P≤0.0001; time: P≤0.01; diet x time: N.S.
Figure 3.7. Mitotic cells in the pyloric caeca of rainbow trout fed the experimental diets for 56 days. Values are mean number of mitotic cells/fold + SEM (6 fish/diet/time; 9 folds/fish where possible). Significance of the linear (L) and quadratic (Q) orthogonal polynomial contrasts of the dependent variable across graded levels of DON from a purified (P) or naturally contaminated (C) source at each sampling time are indicated (* P ≤ 0.025; ** P ≤ 0.01). Diet: P ≤ 0.001; time: P ≤ 0.0001; diet x time: N.S.
Figure 3.8. Mitotic figures in the livers of rainbow trout fed the experimental diets for 56 days. Values are mean number of mitotic figures/field + SEM (6 fish/diet/time; 10 fields/fish). Significance of the linear (L) and quadratic (Q) orthogonal polynomial contrasts of the dependent variable across graded levels of DON from a purified (P) or naturally contaminated (C) source at each sampling time are indicated (* P ≤ 0.025; ** P ≤ 0.01). Diet: P ≤ 0.0001; time: P ≤ 0.0001; diet x time: P ≤ 0.001.
Figure 3.9. Vacuolation scores of the livers of rainbow trout fed the experimental diets for 56 days. Values are mean score/treatment + SEM (6 fish/diet/time). Linear (L) and quadratic (Q) orthogonal polynomial contrasts of the dependent variable across graded levels of DON from a purified (P) or naturally contaminated (C) source at each sampling time were not statistically significant (P>0.025). Significance of the non-orthogonal contrast of Diet 4 (purified, 2.1 ppm DON) vs. Diet 6 (naturally contaminated, 2.1 ppm DON) at each sampling time is indicated (# P≤0.01). Diet: P≤0.0001; time: P≤0.01; diet x time: P≤0.01.
3.5 – Discussion

Two sets of diets, one containing graded levels of purified DON (Diets 1-4) and one containing graded levels of DON from naturally contaminated corn (Diets 5-8), were formulated to determine if the adverse effects of DON-contaminated diets on rainbow trout are solely related to DON, or rather, are the result of other naturally co-occurring mycotoxins and/or their interactions with DON. The dietary inclusion levels of contaminated corn used in the naturally contaminated diets were based on LC-MS/MS analysis of the corn carried out in advance of feed formulation. However, HPLC-MS analysis of the diets containing naturally contaminated corn indicated higher concentrations of DON (2.1, 4.1 and 5.9 ppm) than targeted (1, 2 and 3 ppm) based on the initial LC-MS/MS analysis of the corn. In contrast, the purified diets contained lower concentrations of DON (0.7, 1.4 and 2.1 ppm) than anticipated, possibly due to losses of the purified compound incurred during mixing of the mineral premix (used as a carrier of DON) and the diets.

The reason(s) for the higher concentration of DON in the contaminated corn following diet preparation in comparison to that determined prior to the feed formulation are not clear, but several possibilities exist. This discrepancy may be associated with the use of different analytical techniques (LC-MS/MS vs. HPLC-MS). Substantial inter-laboratory variation in the determination of DON among facilities using official analytical methods has also been documented (Krska et al., 2001). For example, Pettersson (1998) reported poor agreement among several European laboratories (CV=60%) for the determination of DON in a naturally contaminated wheat sample. A number of potential factors responsible for the high variability were identified, including matrix interference and carry-over or memory effects from previous
samples (Pettersson, 1998; Kraska et al., 2001). Alternatively, the differences in the analyzed DON content of the contaminated corn may be related to sampling error associated with the often heterogeneous distribution of mycotoxins in a given commodity lot or to the accumulation of mycotoxins during storage, despite concerted efforts to collect representative samples and maintain adequate storage (i.e. temperature and moisture) conditions (Campbell et al., 1986; Whitaker, 2005). One additional explanation may be the presence of insect activity in the contaminated grain during the period between the initial mycotoxin analysis of the grain and the manufacturing of the experimental diets. Magan et al. (2003) noted that insects play an important role in post-harvest fungal ecology due to the production of metabolic heat which generates water via condensation. Collectively, these issues highlight the complexity of achieving targeted concentrations of mycotoxins in experimental feeds, particularly when naturally contaminated sources are used.

In spite of these limitations, the current results suggested that the adverse effects of the naturally contaminated diets on the performance of rainbow trout were exclusively related to DON and not associated with the presence of other mycotoxins in the grain. This is primarily supported by the lack of significant differences between fish fed Diet 4 (2.1 ppm purified DON) and Diet 6 (2.1 ppm DON from naturally contaminated corn) for weight gain, TGC, FI, FE, carcass composition (water, CP, lipid, ash and GE) and nutrient utilization (RN, RE, NRE and ERE). The effects of the diets containing purified DON on the aforementioned parameters were also in good agreement with our previous observations in rainbow trout fed naturally contaminated diets containing comparable levels of DON (0.3-2.6 ppm) in addition to detectable concentrations of 15-ADON and ZON, each ranging from 0.2 to 0.4 ppm (Hooft et al., 2011).
Likewise, although the naturally contaminated diets contained considerably higher concentrations of DON compared to the purified diets in the current study, the effects of both sets of diets on growth performance, carcass CP content and nutrient utilization of rainbow trout were similar. These results are in contrast to studies in pigs reporting less pronounced effects of diets containing purified DON on feed refusal, feed intake and weight gain compared to diets containing equivalent concentrations of DON from naturally contaminated ingredients (Forsyth et al., 1977; Friend et al., 1986b; Trenholm et al., 1994). The specific mycotoxin contamination profile of the grain used to formulate the naturally contaminated diets in the present study may account for these differences.

HPLC-MS analysis indicated that two of the naturally contaminated diets (Diets 7 and 8) also contained the estrogenic mycotoxin, ZON (0.5 and 0.6 ppm, respectively). Diet 6, in contrast, contained only DON (2.1 ppm). Nonetheless, given that 1.4 ppm 15-ADON and 2.2 ppm ZON were found in the contaminated corn using HPLC-MS, it is likely that all of the naturally contaminated diets contained these mycotoxins, albeit perhaps at levels below the PQL of the HPLC-MS technique employed. Relative to DON, its acetylated precursors, 3-acetyl-DON (3-ADON) and 15-ADON, appear to have similar toxicities in both mice and pigs (Thompson and Wannemacher, 1986; Forsell et al., 1987; Pestka et al., 1987). Abbas et al. (1986) suggested that the presence of 15-ADON in naturally contaminated corn might account, in part, for feed refusal of swine that could not be solely attributed to DON. However, in accordance with our study, no significant interactions between DON (6 ppm) and 15-ADON or 3-ADON (2 ppm) were observed for ADG, feed consumption or FE of 12- to 15-week-old barrows over a 3-week period (Rotter et al., 1992). The apparent absence of an effect of ZON (≤ 0.6 ppm) in the
naturally contaminated diets on the growth performance of rainbow trout was also previously alluded to. Feed intake of juvenile rainbow trout fed a diet containing 3.8 ppm purified DON was significantly less than that of fish fed a diet containing 3.3 ppm DON (and 0.5 ppm ZON) from naturally contaminated corn; however, there were no differences in weight gain, FE or respiratory burst activity of head kidney leucocytes suggesting an absence of interactive effects between DON and ZON (Ryerse et al., 2015). This finding seems to be indirectly supported by Döll et al. (2010) who did not observe any adverse effects of diets containing up to 0.8 ppm ZON on the growth performance or health of Atlantic salmon. Using semi-purified diets, Forsell et al. (1986) were also unable to identify significant synergistic or antagonistic interactive effects of 10 ppm ZON and 5 ppm DON on weight gain, feed intake, terminal organ weights, hematology or serum immunoglobulin (Ig) M, IgG or IgA levels of weanling female mice. The lack of evidence of any direct toxicological interactions between DON and 15-ADON or DON and ZON on growth performance in other species appears to be in good agreement with the results reported here.

The initial LC-MS/MS analysis of the contaminated corn conducted prior to the formulation of the experimental diets indicated notable concentrations of DON-3-glucoside (D3G; 3.1 ppm) and aurofusarin (9.8 ppm). Consequently, the naturally contaminated diets may have contained these metabolites in addition to DON, 15-ADON and ZON, although their presence in the experimental diets was not analyzed. D3G belongs to a group of compounds known as masked mycotoxins, which, as their name implies, elude direct detection by routine chromatographic (e.g. HPLC-MS) and immunochemical-based (e.g. ELISA) analytical techniques (Berthiller et al., 2013). D3G is an issue of increasing concern in human and animal health due
to its frequent co-occurrence with DON (Berthiller et al., 2009b; Desmarchelier and Seefelder, 2011; De Boevre et al., 2012). Nagl et al. (2012, 2014) recently demonstrated the nearly complete hydrolysis of purified D3G in the intestinal tract of both rats and pigs. In theory, intestinal hydrolysis of D3G to DON may potentially increase the total DON load of an individual relative to that present in the feed. However, the oral bioavailability of D3G was reduced by up to a factor of two compared to DON, indicating that it may be of considerably lower toxicological relevance than its native form. Poppenberger et al. (2003) showed that D3G inhibits protein biosynthesis to a far lower extent than DON in wheat ribosomes, but the related consequences have not yet been investigated in mammalian systems. Similarly, the biological importance of aurofusarin, a red pigment produced by numerous Fusarium fungal species, is currently not well-defined (Medentsev et al., 1993; Frandsen et al., 2006). Dvorska et al. (2001) observed significant decreases in vitamins E and A as well as in total carotenoid, lutein and zeaxanthin concentrations and a significant increase in yolk susceptibility to lipid peroxidation in quail eggs obtained from birds fed a diet supplemented with 26.4 ppm aurofusarin. The relevance of these findings to the potential effects of the much lower levels of aurofusarin possibly present in the naturally contaminated diets used here remains unknown. The consistent response of fish fed diets containing either purified or naturally occurring DON suggest that neither D3G nor aurofusarin contributed to the detrimental effects of the experimental diets on the growth performance of rainbow trout to an appreciable extent.

No distinct histopathological lesions were observed in the tissues or organs obtained from fish fed the purified or naturally contaminated diets. Previously, in an unsystematic assessment, we reported subcapsular hemorrhage of the liver of some rainbow trout (IBW = 24
g/fish) fed naturally contaminated diets containing 1.4, 2.0 and 2.6 ppm DON (and 0.3-0.4 ppm 15-ADON and ZON). The same macroscopic lesion was confirmed in one-year-old rainbow trout (IBW ≈ 180 g/fish) after short-term feeding of an experimental diet contaminated with 2 ppm purified DON without any substantial histopathological effects (Matejova et al., 2014). Conversely, in the former study, morphological changes including subcapsular edema, fatty infiltration and phenotypically altered hepatocytes were observed in a number of DON-exposed fish (Hooft et al., 2011). Tola et al. (2015) described comparable histological changes including cytoplasmic vacuolation, subcapsular edema and areas of focal necrosis in the livers of some red tilapia (IBW = 4.3 g/fish) fed diets containing 0.3 to 1.2 ppm from naturally contaminated wheat; however, no significant association between increasing levels of DON and histopathological changes could be discerned using a standardized assessment protocol. The interpretation of histopathological changes is particularly subject to the criteria/scoring system used and this may partially explain such differences among studies (Gibson-Corley et al., 2013; Klopfleisch, 2013). Substantial variability in lesions associated with exposure to feed-borne DON at concentrations within the range of those used in this study has also been reported in other monogastric species (Bergsjø et al., 1993; Goyarts et al., 2005; Awad et al., 2006a,b; Dänicke et al., 2008; Grenier et al., 2011). Nonetheless, using a robust experimental design and a systematic approach to identify potential pathological changes, we observed that the highly significant impact of DON on the growth performance of rainbow trout, at least at the dietary concentrations used here, is not related to marked tissue injury or damage.

In general, increasing concentrations of either purified or naturally occurring DON resulted in significant linear and/or quadratic increases in the number of dead (apoptotic or
necrotic) cells in the pyloric caeca of rainbow trout. In support of these findings, DON-induced immunosuppression has been attributed to apoptosis of lymphoid tissues and macrophages in murine models and cultured cells exposed to high concentrations of DON (Pestka et al., 1994; Yang et al., 2000; Zhou et al., 2000, 2003a, 2005a,b; Islam and Pestka, 2006). Similarly, a relatively high concentration of DON (100 μM) was required to induce significant apoptosis in human intestinal cell lines (Maresca et al., 2002; Sergent et al., 2006). Comparatively, we were able to demonstrate significant increases in the number of dead cells in the intestine of rainbow trout fed diets containing more realistic concentrations of DON. Kolf-Clauw et al. (2009) also reported the presence of numerous apoptotic cells in a pig jejunal explant culture incubated with 5 μM DON, corresponding to an estimated dietary concentration of 1.5 ppm. Nonetheless, to our knowledge, our study represents the first time that significant changes in the number of apoptotic or necrotic cells in the intestine have been quantified in vivo. Rainbow trout fed the naturally contaminated diet containing 2.1 ppm DON had a significantly lower number of dead cells in the pyloric caeca compared to fish fed the diet containing 2.1 ppm purified DON at each sampling time point. This suggests the potential of an antagonistic interactive effect between DON and other mycotoxins present in the naturally contaminated diet (CAST, 2003; Eaton and Gilbert, 2008; Grenier and Oswald, 2011).

In contrast to the relatively consistent increases in the number of dead cells with increasing concentrations of purified or naturally contaminated DON, the effects of the experimental diets on the number of mitotic cells in the pyloric caeca and liver appeared more variable. Decreased cellular proliferation in the intestine and liver of pigs exposed to feed-borne DON has been previously described. For example, feeding a diet containing 2.8 ppm
DON for 35 days significantly reduced the number of mitotic figures in the enterocytes of 5-week-old piglets (Bracarense et al., 2012). A significant reduction in the average number of mitotic figures in the intestinal crypts of the jejunum and ileum was also observed in piglets of the same age following consumption of a diet containing 1.5 ppm DON for 4 weeks (Gerez et al., 2015). Grenier et al. (2011) reported increased development of megalocytosis in weaned piglets fed a diet contaminated with 2.8 ppm DON isolated from F. graminearum. Megalocytes have been described as morphologically and functionally viable cells able to grow, but not divide, a phenomenon consistent with cell cycle arrest and the anti-mitotic effects of DON (Zimmerman, 1999; Svoboda et al., 1971). A significant decrease or a trend towards a significant decrease in the number of mitotic figures in the pyloric caeca and liver of rainbow trout were likewise observed with increasing concentrations of DON at several sampling time points in the present study. Concurrently, after the second week, we also noted a contradictory linear increase in the number of mitotic figures in the liver of fish fed the naturally contaminated diets and a quadratic effect of the purified diets on the number of mitotic cells in the intestine. The significant effect of sampling time on the number of mitotic cells in both the liver and pyloric caeca, and a significant interaction between diet and sampling time on the number of mitotic figures in the liver, may account for these results. Pietsch and Burkhardt-Holm (2015) similarly reported that the severity of alterations in liver histology of carp fed diets contaminated with 0.95 ppm DON was variable depending on the duration of exposure.

The aforementioned changes in cellular metabolism observed in our study are generally consistent with the known molecular mechanisms of action of DON. The involvement of MAPKs in the onset of DON-induced apoptosis via the intrinsic mitochondrial pathway has been
convincingly demonstrated. More specifically, phosphorylation of the p38 MAPK isoform and its downstream substrate, p53, have been shown to increase caspase 3-dependent DNA fragmentation in DON-exposed macrophages and human colon carcinoma cells (Zhou et al., 2005b; Bensassi et al., 2009). Similarly, MAPK-dependent induction of the cyclin-dependent kinase inhibitor p21 has been linked to impaired cell cycle progress in response to DON, which may explain in vivo observations of decreases in the number of mitotic cells (Yang et al., 2008). In addition to MAPK-mediated effects, DON has been shown to affect cellular oxidative status and promote oxidative stress in mammalian and fish in vitro and in vivo models (Rizzo et al., 1994; Kouadio et al., 2005, 2007; Dinu et al., 2011; Sanden et al., 2012; Hou et al., 2013; Pietsch et al., 2014a,b; Pietsch and Burkhardt-Holm, 2015; Šišperová et al., 2015). Sustained perturbation of oxidative balance can significantly impair cell function and may result in cell death (Chandra et al., 2000). However, the potential contribution of DON-induced oxidative stress to alterations in cell death and proliferation remains unclear (Bensassi et al., 2009; Krishnaswamy et al., 2010).

Although we observed a significant effect of diet, time and the interaction of these variables on the degree of hepatic vacuolation, there was no significant association between vacuolation and increasing concentrations of either purified or naturally occurring DON. Vacuolation of the liver may reflect a number of conditions such as accumulation of glycogen, fatty infiltration or hydropic change (Mumford et al., 2007). Ultrastructural examination of liver tissue in gilts and sows fed diets containing 6.1 or 9.6 ppm DON revealed an increase in fatty vacuoles accompanied by a loss of bound ribosomes from the rough ER (Tiemann et al., 2006b, 2008a). A rough ER with intact ribosomes is necessary to produce apolipoproteins, which are
required for the transport of lipids from the liver to the tissues (Zimmerman, 1999; Tiemann et al., 2006b). Consequently, the defective synthesis of apolipoproteins resulting from degranulation of the rough ER may result in fatty vacuolation of the liver. We also attributed vacuolation of the liver of some rainbow trout fed a diet containing 2.6 ppm DON to fatty infiltration in an earlier assessment of selected tissues (Hooft et al., 2011). However, in agreement with the current study, Tola et al. (2015) were unable to observe a significant correlation between cytoplasmic vacuolation of the liver and DON in red tilapia fed diets containing 0.3 to 1.2 ppm DON from naturally contaminated wheat. Similarly, no significant effect of diets containing 0.35, 0.62 and 0.95 ppm DON on the extent of hepatic vacuolation was reported in common carp, despite evidence of fat aggregation in the livers of some DON-exposed groups (Pietsch et al., 2014b). The livers of rainbow trout fed the naturally contaminated diet containing 2.1 ppm DON did have a significantly higher vacuolation score compared to those of fish fed the purified diets after 4 and 6 weeks of the experiment, suggesting that the presence of other compounds in the diet may have adversely affected hepatic metabolism, but further work is necessary to investigate this possibility. Nonetheless, vacuolation score of the liver does not appear to be a particularly meaningful indicator of exposure to DON in rainbow trout which may be related to an inherent high variability in the lipid content of the liver in several species of fish (Mumford et al., 2007).

3.6 – Conclusion

The results of this study suggest that decreased growth performance and nutrient utilization efficiency of rainbow trout exposed to diets formulated using grains naturally
contaminated with DON and other *Fusarium* mycotoxins are most probably associated only with DON. The apparent lack of any interactive effects between DON and other co-occurring mycotoxins (namely ZON and 15-ADON) present in the experimental diets on the performance of rainbow trout is in general agreement with studies conducted in other species. These findings may provide much needed insight for the future development of regulatory or legislative guidelines related to co-contamination of human and animal feeds. A complete and systematic examination of numerous tissues and organs revealed the absence of any distinct pathological lesions associated with increasing, graded concentrations of either purified (0-2.1 ppm) or naturally occurring (0-5.9 ppm) DON. This indicates that extensive tissue damage or injury is not responsible for the highly significant detrimental effects of practically relevant doses (i.e. up to 2 ppm) of DON on the growth performance of rainbow trout. Interestingly, changes in the number of dead cells (apoptotic and/or necrotic) in the intestine and the number of mitotic cells in both the intestine and liver were fairly consistent with the known mechanisms of DON-induced cell death and cell cycle arrest. This appears to be the first time that significant increases in the number of dead cells have been quantified *in vivo* in fish at concentrations of DON relevant to human and animal exposure. Rainbow trout may therefore represent a highly sensitive and appropriate model for continued investigation of the low-dose subclinical cellular and metabolic effects of DON. Notable advantages of rainbow trout as a model species compared to mammalian animal models include a lower per diem cost and the ability to use larger numbers of animals to address statistically challenging questions.
CHAPTER 4 – EVALUATION OF THE EFFICACY OF A COMMERCIAL FEED ADDITIVE AGAINST THE ADVERSE EFFECTS OF FEED-BORNE DEOXYNIVALENOL (DON) ON THE PERFORMANCE OF RAINBOW TROUT (ONCORHYNCHUS MYKISS)

4.1 – Abstract

Rainbow trout are highly sensitive to low, industrially-relevant, levels of the feed-borne Fusarium mycotoxin deoxynivalenol (DON), a common contaminant of plant feedstuffs. Feed additives (yeasts, bacteria, enzymes, etc.) are frequently employed as the primary strategy for counteracting the negative effects of mycotoxins on animal performance. However, their application in aquaculture feeds is still largely speculative. A 12-week growth trial with rainbow trout (initial average body weight=1.8 g/fish) was carried out to assess the efficacy of a commercially available feed additive purportedly effective for preventing the adverse effects of DON. Fish were reared at 16.4 °C and fed a total of eight diets containing low, graded levels of DON (0.3, 1.0, 1.5 and 2.0 ppm) from naturally contaminated corn with or without the commercial feed additive (CFA). Highly significant linear decreases in weight gain (P<0.0001), feed intake (P<0.0001) and thermal-unit growth coefficient (TGC, P<0.0001) were associated with increasing levels of DON in fish fed the diets with or without the CFA. Similarly, regardless of CFA inclusion, rainbow trout fed the experimental diets experienced significant linear and in some cases, quadratic decreases in whole body water (P<0.05), crude protein (CP, P<0.01), lipid (P≤0.05), ash (P<0.05) and gross energy (GE, P<0.01) content. Significant linear or quadratic decreases in retained nitrogen (RN; P<0.0001), recovered energy (RE, P<0.0001), nitrogen retention efficiency (NRE; P<0.05) and energy retention efficiency (ERE, P<0.05) were also observed in response to increasing levels of DON in rainbow trout fed the diets with or without the CFA. There was no significant interaction between DON and CFA (DON x CFA, P>0.05) for
any of the aforementioned parameters indicating that inclusion of the CFA in the diets at the recommended dosage did not alleviate the adverse effects of DON on growth performance and nutrient utilization of rainbow trout. The feed additive used here was developed through extensive research and development with homeotherms; therefore, use in feeds for cold water fish species may be outside the scope of its effectiveness. Additional work is essential to systematically develop novel approaches for mycotoxin mitigation in highly sensitive farmed fish species.

4.2 – Introduction

Mycotoxins are a naturally occurring, chemically and structurally diverse group of more than 300 secondary metabolites produced by the mycelial structure of filamentous fungi, which are characterized by their potential to elicit undesirable effects in humans and animals following consumption of contaminated foods or feedstuffs (Hussein and Brasel, 2001; Bryden, 2007). Mycotoxin contamination resulting from fungal infection of agricultural crops and commodities is closely related to environmental conditions, particularly temperature and moisture, and may occur at various stages of production (in the field, during harvest or processing and in storage; Marquardt, 1996; Whitlow et al., 2010; Whitlow and Hagler, 2010). In general, mycotoxins are chemically and thermally stable, rendering them unsusceptible to routine feed manufacturing processes such as extrusion (Bennett and Richard, 1996). Unspecific symptoms ranging from reduced production efficiency to mortality are associated with consumption of mycotoxin-contaminated feeds, making diagnosis difficult (Whitlow et al., 2010). Moreover, sensitivity to mycotoxins varies greatly between species and is dependent on
several factors which can modify the expression of toxicity, including age, gender and nutritional and health status prior to exposure (Rotter et al., 1996). It has been estimated that 25% of the world’s crop production is contaminated with mycotoxins (CAST, 2003). The economic impact of mycotoxins is effectively impossible to quantify; however, risk assessment analyses have estimated that financial losses to U.S. agriculture associated with crop losses, mitigation efforts and reduced livestock performance or mortality range from $630 million to $2.5 billion per annum (CAST, 2003; Wu, 2004).

The primary classes of mycotoxins with relevance to human health and animal agriculture are produced by fungal species belonging to the genera Aspergillus, Penicillium, and Fusarium. These include aflatoxins, ochratoxins, fumonisins, zearalenone (ZON) and the structurally-related trichothecenes (Marquardt, 1996). The trichothecene mycotoxin, deoxynivalenol (DON), is a ubiquitous contaminant of small cereal grains produced by Fusarium fungal species (Sudakin, 2003; Glenn, 2007). Between 2004 and 2011, analysis of more than 17,000 samples of feeds and feed ingredients collected worldwide indicated that 56% of samples were contaminated with an average of 1 ppm DON (Streit et al., 2013; Schatzmayr and Streit, 2013). This finding was supported by a more recent global survey of more than 4,200 samples of a variety of feedstuffs. Nearly 60% of samples analyzed contained detectable levels of DON; notably, 42% of all surveyed samples were contaminated with DON at levels above 0.3 ppm. Worldwide, average concentrations of DON in samples of whole grain corn, wheat, corn gluten meal (CGM), soybean meal (SBM) and Dried Distillers Grains with Solubles (DDGS) were 0.7, 1.1, 0.9, 0.4 and 1.2 ppm, respectively. Northern Europe and North America had the highest average concentrations of DON in tested samples (1.5 and 1.3 ppm, respectively;
Nährer and Kovalsky, 2014). Nevertheless, globalization of commodity trade and changing climate patterns have made DON an international concern (Wu et al., 2004).

The use of an ever-evolving and expanding number of cost-effective plant ingredients of variable origin and quality necessary to maintain the rapid growth of the aquaculture sector has significantly increased the risk of exposing farmed fish to mycotoxins (Spring and Fegan, 2005; Gatlin et al., 2007). Despite limited availability of information regarding DON contamination of finished fish feeds at present, Pietsch et al. (2013) reported detectable levels of DON in 80% of samples at concentrations up to 0.8 ppm in a small-scale (n = 11) survey of commercial carp feeds originating from central Europe. Relatively lower levels of DON (up to 0.24 ppm) were found in samples of fish feed analyzed in 2014 as part of a monitoring program commissioned by the Norwegian Food Safety Authority (n = 26; Sanden et al., 2015). In the same year, Gonçalves et al. (2016) reported similar average and maximum levels of DON (0.2 and 0.4 ppm, respectively) in shrimp and fish feed samples collected in Asia (n = 31) and Europe (n = 10); however, both surveys indicated a high prevalence (65-68%) of DON at detectable concentrations in the tested samples (Gonçalves et al., 2016; Sanden et al., 2015). Furthermore, a high frequency of multi-mycotoxin occurrence (76%) was observed in samples collected in Asia and Europe (Gonçalves et al., 2016).

Consumption of feedstuffs contaminated with DON results in a variety of adverse effects in farm and laboratory animals (Coulombe, 1993). Chronic feed-borne exposure to low doses of DON is commonly associated with anorexia (reduced feed intake and growth), decreased productivity and altered nutritional efficiency (Rotter et al., 1996; Pestka and Smolinski, 2005). Comparatively, acute, short-term exposure to higher concentrations of DON may result in
leucocytosis, gastrointestinal hemorrhage, diarrhea and emesis or vomiting, particularly in pigs (Forsyth et al., 1977; Young et al., 1983; Pestka et al., 1987; Pestka, 2010a). The toxicity of DON is primarily related to its ability to inhibit protein synthesis in rapidly proliferating cells and tissues (Ehrlich and Daigle, 1987; Zhou et al., 2003a; Li and Pestka, 2008), regulate MAPK-dependent cellular processes including cytokine production (Moon and Pestka, 2003; Kinser et al., 2004), apoptosis (Zhou et al., 2005a,b; Bensassi et al., 2009) and mitosis (Tiemann et al., 2003; Yang et al., 2008) and alter neuroendocrine and enteroendocrine signaling within the gut-brain axis related to anorectic or emetic behaviours (Fitzpatrick et al., 1988; Prelusky et al., 1992; Fioramonti et al., 1993; Swamy et al., 2004; Flannery, 2012).

Current evidence indicates that some commercially important finfish species are extremely sensitive to DON. Highly significant decreases in weight gain, thermal-unit growth coefficient (TGC), feed intake (FI), feed efficiency (FE), carcass crude protein (CP) content, retained nitrogen (RN), recovered energy (RE), nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were reported in rainbow trout fed a series of diets containing increasing, graded levels of DON (0.3, 0.8, 1.4, 2.0 and 2.6 ppm) from naturally contaminated corn (Hooft et al., 2011). Similar reductions in growth performance and nutrient utilization efficiencies were observed in fish fed diets containing naturally occurring (2.1, 4.1 and 5.9 ppm) or purified (2.1, 4.1 and 5.9 ppm) DON in Chapter 3. Fish pair-fed a control diet (0.3 ppm DON) had significantly higher TGC, FE, whole body crude CP content, RN, NRE and ERE compared to their counterparts fed the diet containing 2.6 ppm DON, suggesting that the effects of DON on the performance of rainbow trout are not simply due to reduced feed intake, but rather, related to deleterious metabolic effects (Hooft et al., 2011). More recently, Tola et al. (2015)
observed significant linear decreases in weight gain, TGC, FI and FE of juvenile red tilapia (Oreochromis niloticus × O. mossambicus) fed diets containing approximately 0.07, 0.3, 0.5, 0.9 and 1.2 ppm DON from naturally contaminated wheat. These studies highlight the importance of evaluating potential solutions to minimize the negative impacts of mycotoxins on fish performance in sensitive species.

Feed additives are routinely incorporated into feeds to mitigate the effects of mycotoxins on animal health and performance. Anti-mycotoxin feed additives can be broadly categorized into adsorbing agents and biotransforming agents. Mycotoxin adsorbents or binders such as clay minerals or yeast cell wall extracts reduce the bioavailability and subsequent uptake and systemic distribution of mycotoxins to target organs, while biotransforming agents (e.g. bacteria, fungi, yeast and enzymes) are aimed at the degradation of parent compounds (i.e. mycotoxins) into non- or less-toxic metabolites in the gastrointestinal tract (Boudergue et al., 2009). The efficacy of many different commercially available feed additives in preventing negative growth and health related effects of mycotoxins has been the subject of numerous studies in several terrestrial monogastric species, often with contradictory results (Girish and Smith, 2008; Leung et al., 2007; Swamy et al., 2002a,b, 2003; Dänicke et al., 2004b; Cheng et al., 2006). To date, the majority of efforts aimed at evaluating the effectiveness of anti-mycotoxin feed additives in fish have focused on the use of clay mineral-based products in the prevention of aflatoxicoses in rainbow trout (Ellis et al., 2000; Abdel-Wahhab et al., 2005; Zychowski et al., 2013; Selim et al., 2014). However, little work has been conducted to evaluate the use of adsorbing and/or biotransforming agents in fish feeds naturally contaminated with DON and other common Fusarium mycotoxins. The objective of
this study was, therefore, to investigate the potential efficacy of a commercial feed additive (CFA) with adsorbing and biotransforming properties in minimizing or preventing the adverse effects of diets naturally contaminated with DON on growth performance and nutrient utilization of rainbow trout.

4.3 – Materials and methods

4.3.1 – Fish husbandry and experimental conditions

Rainbow trout were obtained from the Alma Aquaculture Research Station (Elora, ON, Canada). Subsequently, groups of 50 fish with an initial average body weight of 1.8 g/fish were randomly distributed into 24 tanks (3 replicates per treatment). Fish were maintained in a flow-through system consisting of 60 L fibreglass tanks, individually aerated and continuously supplied with well water at a rate of 3 L/min. Water temperature was maintained at 16.4 ± 1.4 °C and photoperiod was set at a ratio of 12 hours light: 12 hours dark in a windowless laboratory. Prior to the start of the growth trial, fish were acclimated to the experimental conditions for approximately two weeks, during which time they were fed a commercial trout feed (Martin Mills Inc., Elmira, ON, Canada). Throughout the duration of the experiment, the animals were kept in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984) and the University of Guelph Animal Care Committee.

Feed intake was recorded weekly and individual tanks of fish were weighed every 28 days. A pooled sample of 30 fish was taken for analysis of initial carcass proximate composition prior to the start of the experiment. After the 12-week experimental period, 14 fish per tank
were randomly sampled for determination of final carcass composition. Fish were humanely euthanized by an overdose of tricaine methane sulfonate (200 mg/L water).

4.3.2 – Experimental diets and feeding protocol

The formulation of the experimental diets is shown in Table 4.1. Two sources of ground corn (< 2 mm) were acquired from the Arkell Feed Mill (University of Guelph, Guelph, ON, Canada). All diets contained a total inclusion of 25% corn. Incremental increases (0, 8, 16 and 24%) in the percentage of naturally contaminated corn (10 ppm DON) and concurrent decreases (25, 17, 9 and 1%) in the amount of uncontaminated corn (0.9 ppm DON) were used to formulate two sets of diets, each containing four graded levels of DON. A commercial feed additive (CFA) was incorporated into one set of the experimental diets at a recommended inclusion rate of 2 g/kg diet (Diets 2, 6, 7 and 8). Diets 1, 3, 4 and 5 were formulated to contain the same levels of DON as Diets 2, 6, 7 and 8, respectively, without the CFA. Diets were mixed using a Hobart mixer (Hobart Ltd., Don Mills, ON, Canada), steam pelleted using a laboratory pellet mill (California Pellet Mill Co., San Francisco, CA, USA), dried under forced-air at room temperature for 24 hours, sieved and stored at 4 °C until used. Fish were hand-fed to satiety three times daily on weekdays and once per day on weekends.
Table 4.1. Formulation of the experimental diets and dietary mycotoxin concentrations.

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<th>Ingredient (g/100 g diet)</th>
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<td>Fish meal, herring</td>
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<td>Poultry by-products meal</td>
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<td>9.0</td>
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<tr>
<td>Blood meal, porcine, spray-dried</td>
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<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
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<tr>
<td>Corn, clean</td>
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<td>25.0</td>
<td>17.0</td>
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<td>1.0</td>
<td>17.0</td>
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<tr>
<td>Corn, contaminated</td>
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<td>-</td>
<td>8.0</td>
<td>16.0</td>
<td>24.0</td>
<td>8.0</td>
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<tr>
<td>Soy protein concentrate 300 ε</td>
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<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
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<tr>
<td>Biolys®, 52 % lysine</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<td>DL-methionine</td>
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<td>0.04</td>
<td>0.04</td>
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<tr>
<td>Vitamin premix d</td>
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<td>1.0</td>
<td>1.0</td>
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<td>Mineral premix e</td>
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<td>Soybean oil</td>
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<tr>
<td>CFA</td>
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</tr>
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**Analyzed composition (dry matter basis)**

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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
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</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>96.8</td>
<td>96.6</td>
<td>96.5</td>
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<td>96.5</td>
<td>96.6</td>
<td>96.7</td>
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<tr>
<td>Crude protein (%)</td>
<td>40.2</td>
<td>40.3</td>
<td>41.0</td>
<td>41.0</td>
<td>41.3</td>
<td>41.1</td>
<td>41.2</td>
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<tr>
<td>Lipid (%)</td>
<td>23.2</td>
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<td>23.0</td>
<td>22.9</td>
<td>22.9</td>
<td>22.9</td>
<td>22.9</td>
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<tr>
<td>Ash (%)</td>
<td>7.7</td>
<td>8.2</td>
<td>7.6</td>
<td>7.6</td>
<td>8.0</td>
<td>8.4</td>
<td>8.1</td>
<td>7.7</td>
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<tr>
<td>Gross energy (KJ/g)</td>
<td>23.2</td>
<td>23.2</td>
<td>23.2</td>
<td>23.2</td>
<td>23.1</td>
<td>23.1</td>
<td>23.0</td>
<td>22.9</td>
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</table>

**Analyzed mycotoxin concentrations (ppm)**

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<th>6</th>
<th>7</th>
<th>8</th>
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</thead>
<tbody>
<tr>
<td>Deoxynivalenol (DON)</td>
<td>0.3</td>
<td>0.3</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>0.8</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>15-acetyl DON (15-ADON)</td>
<td>ND检察官</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Zearalenone (ZON)</td>
<td>ND检察官</td>
<td>ND</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>ND</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

- Contains: 0.9 ppm deoxynivalenol (DON).
- Contains: 10.0 ppm DON; 1.0 ppm 15-acetyl DON; 1.2 ppm zearalenone (ZON).
- HP 300 (Hamlet Protein, Horsens Denmark).
- Provides per kg of diet: retinyl acetate (vitamin A), 75 mg; cholecalciferol (vitamin D3), 300 mg; menadione Na-bisulfate (vitamin K), 1.5 mg; cyanocobalamine (vitamin B12), 30 mg; D-biotin, 210 mg; choline chloride, 3448 mg; folic acid, 1.5 mg; niacin, 15 mg; calcium-d-pantothenate, 33 mg; pyridoxine–HCl, 7.5 mg; riboflavin, 9 mg; thiamin–HCl, 1.5 mg (Martin Mills Inc., Elmira, ON, Canada).
- Provides per kg of diet: sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; potassium iodine (KI, 24%K, 76%I), 10.5 mg; ferrous sulphate (FeSO₄, 7H₂O, 20%Fe), 65 mg; manganese sulphate (MnSO₄, 36%Mn) 88.9 mg; zinc sulphate (ZnSO₄·7H₂O, 40%Zn), 150 mg; copper sulphate (CuSO₄·5H₂O, 25%Cu), 28 mg; sodium Selenite (Na₂SeO₃, 45.66% Se), 0.7 mg (Martin Mills Inc., Elmira, ON, Canada).
- ND=not detectable.
4.3.3 – Mycotoxin analysis

Samples of the two sources of corn and the eight experimental diets were analyzed for DON, 3-acetyl DON (3-ADON), 15-acetyl DON (15-ADON), nivalenol, T-2 toxin, iso T-2 toxin, acetyl-T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, fusarenone-X, diacetoxydeoxynivalenol (DAS), scirpentriol, 15-acetoxydeoxynivalentriol, neosolaniol, zearalenone (ZON) and zearalenol by gas chromatography (GS)/mass spectrometry (MS). Aflatoxin B₁ (AFB₁) and fumonisin B₁ (FB₁) were analyzed by high performance liquid chromatography (HPLC) and fluorescence detection (Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, ND, USA). The practical quantitation limit (PQL) for all mycotoxins was 0.5 ppm, with the exception of AFB₁ (0.02 ppm) and FB₁ (2 ppm).

4.3.4 – Chemical analysis

Diets, ingredients (included in the diet at more than 3%) and carcass samples were analyzed for dry matter (DM) and ash according to AOAC (1995), crude protein (CP, %N × 6.25) by LECO (LECO Corp., St. Joseph, MI, USA), lipids with an Ankom XT20 fat analyzer (Ankom Technology, Macedon, NY, USA) using petroleum ether and gross energy (GE) content using a Parr 1271 automated bomb calorimeter (Parr Instruments, Moline, IL, USA). In preparation for analysis, diet and ingredients were ground to a fine, homogenous consistency. Fish analyzed for carcass composition were cooked in an autoclave, ground into a homogenous slurry using a food processor, freeze dried, reground and stored at –20 °C until proximate analysis.
4.3.5 – Calculations

Growth rate, expressed as thermal-unit growth coefficient (TGC), was calculated for each tank as: \[ TGC = 100 \times \left( \frac{FBW^{1/3} - IBW^{1/3}}{\text{sum } T \times D} \right), \]
where: FBW = final body weight (g/fish); IBW = initial body weight (g/fish); and sum T × D = sum degrees Celsius × days.

Feed efficiency (FE, gain: feed) was calculated for each tank as: \[ FE = \frac{\text{live body weight gain}}{\text{dry feed intake}}, \]
where: feed intake (FI) = total dry feed/number of fish; and live body weight gain = (FBW/final number of fish) – (IBW/initial number of fish).

Retained nitrogen (RN, g/fish) and recovered energy (RE, KJ/fish) were calculated for each tank as: \[ RN = (FBW \times N \text{ content}_{\text{final}}) - (IBW \times N \text{ content}_{\text{initial}}) \]
and \[ RE = (FBW \times GE \text{ content}_{\text{final}}) - (IBW \times GE \text{ content}_{\text{initial}}), \]
respectively, where: \( N \text{ content}_{\text{final}} \) = nitrogen content (%) of the final carcass sample; \( N \text{ content}_{\text{initial}} \) = nitrogen content (%) of the initial carcass sample; \( GE_{\text{final}} \) = gross energy (KJ/g) content of the final carcass sample; and \( GE_{\text{initial}} \) = gross energy (KJ/g) content of the initial carcass sample.

Nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were calculated for each tank as a percentage of ingested nitrogen (IN, g/fish) and ingested energy (IE, KJ/fish), respectively: \[ \text{NRE (\% IN)} = \left[ \frac{\left( FBW \times N \text{ content}_{\text{final}} \right) - \left( IBW \times N \text{ content}_{\text{initial}} \right)}{\text{IN}} \right] \times 100 \]
and \[ \text{ERE (\% IE)} = \left[ \frac{\left( FBW \times GE \text{ content}_{\text{final}} \right) - \left( IBW \times GE \text{ content}_{\text{initial}} \right)}{\text{IE}} \right] \times 100. \]

4.3.6 – Statistical analysis

All data were analyzed as a 2 × 4 randomized incomplete block design using the PROC MIXED procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, USA), in which DON and CFA were the treatment factors. A DON × CFA interaction term was included in the model and
block was included as a random effect. Tank was considered the experimental unit. The Shapiro-Wilk test in PROC UNIVARIATE of SAS was used to assess normality of all dependent variables prior to other statistical analysis. A log transformation was adopted for variables with a non-normal distribution: weight gain, FI, RN and RE. Similarly, the Brown and Forsythe test was used to test for homogeneity of variances for all dependent variables. Linear and quadratic orthogonal polynomial contrasts across levels of DON with and without CFA were performed for each dependent variable with contrast coefficients generated to match observed DON concentrations using the PROC IML procedure of SAS. Significance was declared at P≤0.05.

4.4 – Results

4.4.1 – Mycotoxin concentrations

A concentration of 0.9 ppm DON was found in the uncontaminated source of corn, while the contaminated corn contained 10.0 ppm DON, 1.0 ppm 15-ADON and 1.2 ppm ZON. Diets 1 (control), 3, 4 and 5 were determined to contain 0.3, 1.0, 1.5 and 2.0 ppm DON, respectively. Similar findings were obtained for the diets including the CFA: analytical results indicated DON concentrations of 0.3, 0.8, 1.5 and 2.0 ppm in Diets 2 (control), 6, 7 and 8, respectively. Detectable concentrations of ZON ranging from 0.3 to 0.5 ppm were also noted in some of the experimental diets (Table 4.1). In order to evaluate the main effects of DON and CFA and their interaction (DON × CFA), a concentration of 1.0 ppm DON for Diet 6 (analyzed value of 0.8 ppm DON) was used for the statistical analysis of all dependent variables.
4.4.2 – Growth performance

Figure 4.1 depicts the similarity in growth curves of rainbow trout fed the experimental diets without (Figure 4.1 A) and with (Figure 4.1 B) the CFA. Overall, weight gain, growth rate (expressed as TGC) and FI were significantly affected by dietary DON concentration (P<0.0001). The inclusion of the CFA in the diets did not have a significant effect on any of the growth performance parameters. Likewise, no significant interaction of DON and CFA on weight gain, TGC or feed intake was observed. Highly significant linear decreases (P<0.0001) in weight gain, TGC (Figure 4.2) and FI were observed in fish fed diets containing increasing, graded levels of DON both without and with CFA (Table 4.2). The significant quadratic decrease (P<0.01) in FE of fish fed the diets without CFA was not present in fish fed diets containing CFA. However, FE of fish fed both sets of diets was numerically similar, ranging from 1.09 to 1.07 without the CFA and 1.10 to 1.07 with the CFA as the level of DON increased from 0.3 to 2.0 ppm. Mortality was not significantly affected by the experimental diets.
Table 4.2. Weight gain, thermal-unit growth coefficient, feed intake, feed efficiency and mortality of rainbow trout (initial average weight=1.8 g/fish) fed the experimental diets for 84 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>CFA (g/kg diet)</th>
<th>Gain (g/fish)</th>
<th>TGC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Feed intake (g/fish)</th>
<th>FE&lt;sup&gt;b&lt;/sup&gt; (gain/feed)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>-</td>
<td>34.5</td>
<td>0.152</td>
<td>31.7</td>
<td>1.09</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>-</td>
<td>27.8</td>
<td>0.136</td>
<td>24.9</td>
<td>1.12</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>-</td>
<td>21.0</td>
<td>0.118</td>
<td>18.6</td>
<td>1.12</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>-</td>
<td>17.9</td>
<td>0.108</td>
<td>16.6</td>
<td>1.07</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Significance<sup>c</sup>

| Linear | P<0.0001 | P<0.0001 | P<0.0001 | N.S. | N.S. |
| Quadratic | N.S. | N.S. | N.S. | P<0.01 | N.S. |

2 0.3 2 36.9 0.157 33.5 1.10 0.0
6 1.0 (0.8) 2 24.0 0.127 22.1 1.08 0.0
7 1.5 2 20.5 0.117 19.1 1.08 0.7
8 2.0 2 16.4 0.102 15.2 1.07 0.0

Significance<sup>d</sup>

| Linear | P<0.0001 | P<0.0001 | P<0.0001 | N.S. | N.S. |
| Quadratic | N.S. | N.S. | N.S. | N.S. | N.S. |

Significance<sup>e</sup>

| DON | P<0.0001 | P<0.0001 | P<0.0001 | N.S. | N.S. |
| CFA | N.S. | N.S. | N.S. | N.S. | N.S. |
| DON \times CFA | N.S. | N.S. | N.S. | N.S. | N.S. |
| S.E.M.<sup>f</sup> | 1.48 | 0.004 | 1.36 | 0.01 | 0.5 |

<sup>a</sup>TGC=thermal-unit growth coefficient.

<sup>b</sup>FE=feed efficiency.

<sup>c</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON without CFA.

<sup>d</sup>Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON with CFA.

<sup>e</sup>Significance of the main effects.

<sup>f</sup>S.E.M.=standard error mean.

<sup>g</sup>N.S.=not statistically significant (P>0.05).
Figure 4.1. Growth curves of rainbow trout (IBW=1.8 g/fish) fed the experimental diets without (A) or with (B) a commercial feed additive (CFA) for 84 days.
Figure 4.2. Growth rate (TGC) of rainbow trout (IBW=1.8 g/fish) fed the experimental diets without (-) or with (+) a commercial feed additive (CFA) for 84 days.
4.4.3 – Carcass composition and nutrient utilization efficiency

The level of DON in the diets had a significant effect on whole body content of water, CP (P<0.001), lipid (P<0.01), ash (P<0.05) and GE (P<0.0001) (Table 4.3). Interestingly, decreases in CP and lipid and a concurrent increase in carcass water content were associated with increasing graded levels of DON (Figure 4.3). Among the carcass composition parameters, only final carcass content of ash was significantly affected (P<0.05) by inclusion of CFA in the diets. However, ash content decreased linearly (P<0.05) with increasing levels of DON regardless of CFA inclusion (Table 4.3). There was no significant interaction of DON and CFA for any of the dependent variables related to carcass composition. Carcass CP content decreased linearly with increasing levels of DON in the diets without (P<0.01) and with (P<0.001) CFA. Lipid and gross energy content of the whole body decreased linearly (P<0.01 and P<0.001, respectively) and quadratically (P<0.01) as the concentration of DON increased in the diets without CFA. Likewise, significant linear decreases in carcass lipid (P<0.05) and gross energy (P<0.01) composition were associated with graded levels of DON in diets containing CFA (Table 4.3).

RN, RE, NRE and ERE were significantly affected (P<0.0001) by DON. CFA and the interaction of DON and CFA had no significant effect on nutrient utilization (Table 4.4). RN and RE decreased linearly (P<0.0001) with increasing levels of DON (without and with CFA). NRE decreased linearly (P<0.001) in response to both sets of diets and a quadratic decrease in NRE (P<0.05) was also noted in fish fed the diets without CFA (Table 4.4; Figure 4.4). Significant linear and quadratic decreases in ERE were observed in fish fed the diets formulated without (linear: P<0.01; quadratic: P<0.001) and with (linear: P<0.01; quadratic: P<0.05) CFA (Table 4.4; Figure 4.5).
Table 4.3. Chemical body composition of the whole carcass of rainbow trout (initial average weight=1.8 g/fish) fed the experimental diets for 84 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>CFA (g/kg diet)</th>
<th>H₂O (%)</th>
<th>CPᵃ (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>GEᵇ (KJ/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>-</td>
<td>69.4</td>
<td>14.7</td>
<td>13.4</td>
<td>2.6</td>
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<tr>
<td>3</td>
<td>1.0</td>
<td>-</td>
<td>69.2</td>
<td>14.6</td>
<td>14.0</td>
<td>2.4</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>-</td>
<td>70.3</td>
<td>14.0</td>
<td>13.4</td>
<td>2.3</td>
<td>8.8</td>
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<tr>
<td>5</td>
<td>2.0</td>
<td>-</td>
<td>71.6</td>
<td>13.9</td>
<td>11.8</td>
<td>2.4</td>
<td>8.1</td>
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Significance⁽ᶜ⁾

<table>
<thead>
<tr>
<th></th>
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</thead>
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<tr>
<td></td>
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<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td>P&lt;0.05</td>
<td>P&lt;0.001</td>
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<tr>
<td></td>
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<td>P&lt;0.01</td>
<td>N.S.</td>
<td>P&lt;0.01</td>
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</tbody>
</table>

Significance⁽ᵈ⁾

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.01</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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</table>

Significance⁽ᵉ⁾

<table>
<thead>
<tr>
<th></th>
<th>DON</th>
<th>CFA</th>
<th>DON × CFA</th>
<th>S.E.M.ᶠ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td>N.S.</td>
<td>N.S.</td>
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</tr>
<tr>
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<td>N.S.</td>
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<tr>
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<td>N.S.</td>
<td>N.S.</td>
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<tr>
<td></td>
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<td>N.S.</td>
<td>N.S.</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.0001</td>
<td>N.S.</td>
<td>N.S.</td>
<td>0.14</td>
</tr>
</tbody>
</table>

ᵃCP=crude protein.
ᵇGE=gross energy.
ᶜSignificance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON without CFA.
ᵈSignificance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON with CFA.
ᵉSignificance of the main effects.
ᶠS.E.M.=standard error mean.
ᵍN.S.=not statistically significant (P>0.05).
Figure 4.3. Whole body (carcass) crude protein (▲), lipid (●) and (■) water content of fish fed the experimental diets containing increasing levels of DON from naturally contaminated corn. Values are overall means of tanks fed diets without and with CFA (n=6) ± SEM.
Table 4.4. Retained nitrogen, recovered energy, nitrogen retention efficiency and energy retention efficiency of rainbow trout (initial average weight=1.8 g/fish) fed the experimental diets for 84 days.

<table>
<thead>
<tr>
<th>Diet</th>
<th>DON (ppm)</th>
<th>CFA (g/kg diet)</th>
<th>RN(^a) (g/fish)</th>
<th>RE(^b) (KJ/fish)</th>
<th>NRE(^c) (% IN)</th>
<th>ERE(^d) (% IE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>-</td>
<td>0.8</td>
<td>316</td>
<td>38.6</td>
<td>41.6</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>-</td>
<td>0.7</td>
<td>260</td>
<td>38.6</td>
<td>43.4</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>-</td>
<td>0.5</td>
<td>189</td>
<td>37.4</td>
<td>42.2</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>-</td>
<td>0.4</td>
<td>149</td>
<td>35.2</td>
<td>37.5</td>
</tr>
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</table>

Significance\(^e\)

<table>
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<th>Quadratic</th>
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<tr>
<td></td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>N.S.(^i)</td>
</tr>
<tr>
<td>6</td>
<td>1.0 (0.8)</td>
<td>N.S.</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Significance\(^f\)

<table>
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<tr>
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<th>Quadratic</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>P&lt;0.0001</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.0001</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.0001</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

Significance\(^g\)

<table>
<thead>
<tr>
<th></th>
<th>DON</th>
<th>CFA</th>
<th>DON × CFA</th>
<th>S.E.M.(^h)</th>
</tr>
</thead>
<tbody>
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<td>N.S.</td>
<td>N.S.</td>
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<td>N.S.</td>
<td>N.S.</td>
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<td>N.S.</td>
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<td>N.S.</td>
<td>N.S.</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\(^a\)RN=retained nitrogen.
\(^b\)RE=recovered energy.
\(^c\)NRE (%IN)=nitrogen retention efficiency (% ingested nitrogen).
\(^d\)ERE (%IE)=energy retention efficiency (% ingested energy).
\(^e\)Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON without CFA.
\(^f\)Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets containing graded levels of DON with CFA.
\(^g\)Significance of the main effects.
\(^h\)S.E.M.=standard error mean.
\(^i\)N.S.=not statistically significant (P>0.05).
Figure 4.4. Nitrogen retention efficiency (NRE) as a percentage of ingested nitrogen (% IN) of rainbow trout (IBW=1.8 g/fish) fed the experimental diets without (-) or with (+) a commercial feed additive (CFA) for 84 days.
Figure 4.5. Energy retention efficiency (ERE) as a percentage of ingested energy (% IE) of rainbow trout (IBW=1.8 g/fish) fed the experimental diets without (-) or with (+) a commercial feed additive (CFA) for 84 days.
4.5 – Discussion

The primary objective of this study was to assess the efficacy of an anti-mycotoxin CFA (with adsorbent and biotransforming properties) for minimizing or preventing the previously documented adverse effects of the common feed-borne contaminant DON on growth and nutrient utilization of rainbow trout. The significant linear and/or quadratic decreases in weight gain, TGC, FI, CP, RN, RE, NRE and ERE of fish in the current study in response to increasing dietary concentrations of DON (0.3, 1.0, 1.5 and 2.0 ppm) were in good agreement with earlier results in rainbow trout fed diets containing 0.3, 0.8, 1.4, 2.0 and 2.6 ppm DON from naturally contaminated corn (Hooft et al., 2011). In contrast to the quadratic decrease in FE of fish fed the diets without CFA, FE of rainbow trout fed the diets containing CFA was not significantly affected by increasing dietary concentrations of DON. Nonetheless, no significant effects of CFA or the interaction between DON and CFA were observed for any of the aforementioned parameters, indicating that inclusion of CFA in the experimental feeds at the recommended rate (2 g CFA/kg feed) did not alleviate the detrimental effects of DON on growth performance or measures of nutrient retention in rainbow trout.

The significant decreases in carcass lipid, ash and GE content and a significant increase in whole body water content reported here are in disagreement with the nonsignificant effects of DON on these body composition indices in our initial study (Hooft et al., 2011). The reasons for these discrepancies are unclear, but the more pronounced effects of DON on carcass composition in the current study may be related to the lower initial body weight of the animals (1.8 g/fish vs. 24.3 g/fish) or the longer duration of the experimental period (84 vs. 56 days). The inverse relationship between relative body lipid and water content has been well-defined;
conversely, the relationship between CP and body water content is usually described as a positive association (Dumas et al., 2007). However, Salze et al. (2014) demonstrated that body composition of rainbow trout fingerlings (1.3 g) differs compared to juvenile (70 g) fish in response to feed restriction: fingerlings exhibited decreased whole carcass CP and lipid contents and a concurrent increase in body water content, whereas juveniles maintained CP content while lipid content decreased and water content increased. The preferential accretion of specific structural proteins in the white muscle of feed restricted juveniles as well as prioritization of hypertrophy in favour of increased hyperplasia in fingerlings in response to decreased FI further support the influence of nutritional status on body composition (Salze et al., 2014). The increase in body water mass despite a significant decrease in carcass CP content in fish fed increasing, graded levels of DON may therefore indicate the conservation of proteins with a high degree of affinity for water. The lack of significant effects of CFA or the interaction between DON and CFA on whole body water, lipid and GE contents indicate that the product did not prevent any of the DON-associated changes in these body composition parameters in rainbow trout. Ash content was significantly affected by CFA inclusion, but the interaction between DON and CFA was not significant, denoting that differences in the ash content of fish fed the diets with or without CFA were independent of DON contamination of the feeds.

It is important to note that in addition to DON, the experimental diets contained 0.3 to 0.5 ppm zearalenone (ZON). Based on analysis of the contaminated corn, the diets may have also contained an acetylated precursor of DON, 15-ADON, albeit at non-detectable concentrations. It is possible that interactions between co-occurring mycotoxins may have contributed to the ineffectiveness of the CFA. However, our observations of comparable
responses of rainbow trout to diets containing either purified DON or DON from naturally contaminated corn, also contaminated with ZON and 15-ADON (Chapter 3), suggest that the existence of mycotoxin interactions is not a plausible explanation for the inefficacy of the CFA. Similarly, no toxicological interactions between DON and ZON or DON and 15-ADON on growth performance have been reported in mice (Forsell et al., 1986) or pigs (Côté et al., 1985; Rotter et al., 1992). Moreover, the additive used here is reportedly effective against a broad-spectrum of mycotoxins including trichothecenes and ZON. Nonetheless, the potential influence of unknown, undetected or unanalyzed compounds (e.g. masked mycotoxins such as glucose conjugates) on the efficacy of the CFA cannot be discounted (Berthiller et al., 2005; Krška and Molinelli, 2007; Sulyok et al., 2007).

The development of mycotoxin adsorbents and biotransforming agents is generally conducted using in vitro models simulating the gastrointestinal tract of homeothermic species (i.e. mammals and birds). Notably, the incubation temperature used in studies aimed at validating efficacy is usually 37 to 39 °C with reference to the normal porcine and avian physiological states (Fuchs et al., 2000, 2002; Avantaggiato et al., 2004). Such temperatures are in substantial contrast to the internal temperature of poikilotherms which is subject to environmental variation. Water temperature in the current study was maintained within a normal range for rainbow trout (approximately 16.4 °C), which may have negatively affected the functional absorptive and detoxifying properties of the CFA. Furthermore, Yiannikouris et al. (2004a,b, 2006) reported an absence of affinity of β-1,6-glucans derived from the cell wall of *Saccharomyces cerevisiae* for AFB₁ and DON in alkaline conditions (pH=8.0) and optimal binding of AFB₁, DON, ZON and ochratoxin A (OTA) at a pH of 6.0. This is comparable to the average pH
values of 6.3 and 6.4 in the proximal small intestine of pigs (Merchant et al., 2011) and broiler chickens (Mabelebele, 2013), respectively, but is lower than the more basic pH conditions of the caecal intestine (6.7-7.8) and post-caecal small intestine (7.1-8.1) of rainbow trout (Fard et al., 2007). Consequently, we hypothesize that the temperature and pH or other dynamic conditions such as the rate of mycotoxin absorption, intestinal transit time and peristaltic movements in the gastrointestinal tract of rainbow trout may have negatively influenced the effectiveness of the CFA. In support, Döll et al. (2004) concluded that their observations concerning the effects of incubation time and pH on the efficacy of feed additives in vitro demonstrate the necessity to adjust the conditions of the in vitro system to the physiological conditions of the target animal species.

Considerable research has been conducted to evaluate the efficacy of yeast cell wall-derived glucomannan polymers and microbial or enzymatic detoxifying agents in preventing fusariotoxicoses in a number of monogastric species including broiler chickens (Swamy et al., 2002a; Dänicke et al., 2003; Awad et al., 2012), pigs (He et al., 1993; Swamy et al., 2002b, 2003; Dänicke et al., 2004b; Cheng et al., 2006), laying hens (Dänicke et al., 2002; Chowdhury and Smith, 2004, 2005) and turkey poults (Girish et al., 2008; Girish and Smith, 2008) with highly variable species- and endpoint-dependent results. In addition, Leung et al. (2007) proposed that the lack of efficacy of a glucomannan adsorbent (GMA) in preventing the detrimental effects of diets naturally contaminated with 3.9 ppm DON, 0.4 ppm 15-ADON, 0.4 ppm ZON and 9.9 ppm fusaric acid on body weight, blood pressure, serum protein content and serum enzyme activities in mature female dogs could be related to a sub-optimal dose of GMA resulting from decreased FI. The addition of CFA to the diets in our study was based on the recommended
inclusion rate provided by the manufacturer (2 g CFA/kg feed). The evaluation of different feed additive doses may be useful, particularly in fish, since recommended inclusion rates are frequently based on applications in terrestrial species. Similarly, the implementation of pair-feeding treatments to eliminate the potentially confounding effect of reduced FI on the efficacy of the CFA and the use of additional endpoints (such as hematological and serum biochemical parameters) may be valuable to better understand the usefulness of anti-mycotoxin agents in fish feeds.

Thus far, much of the published work regarding the effectiveness of anti-mycotoxin feed additives in fish has been limited to the use of adsorbents to alleviate the toxicity of AFB₁. Overall, relatively good success has been achieved with the use of clay minerals and yeast or yeast cell-wall constituents in counteracting the negative effects of AFB₁ or its precursor sterigmatocystin (Stg) on indicators of growth, health and metabolism in rainbow trout (Ellis et al., 2000) and omnivorous warm water species (Abdel-Wahhab et al., 2005; Agouz and Anwer, 2011; Selim et al., 2014). These observations are in accordance with the high affinity and capacity of clay minerals for aflatoxins, which is in contrast to their relative ineffectiveness against more non-polar mycotoxins of practical significance, including fusariotoxins (Huwig et al., 2001). However, Zychowski et al. (2013) were not able to substantiate the efficacy of a montmorillonite clay product (NovaSil™) with regard to prevention of the adverse effects of AFB₁ (1.5 or 3 ppm) on weight gain, FE, hepatosomatic index (HSI) or hepatic lesions of juvenile Nile tilapia. The authors proposed that the lack of efficacy of the product used in their study may have been related to feed manufacturing (i.e. mixing, pelleting) or interactions between feed components and the negatively-charged interlayer of the additive which rendered it less
capable of sequestering AFB$_1$ or interfered with the availability of essential nutrients. The latter suggestion is a well-known limitation of clay silicates (Moshtaghian et al., 1991; Jouany, 2007). Nonetheless, the comparable response of rainbow trout to the control diets (0.3 ppm DON) regardless of CFA inclusion in the present study suggests that the adsorbent component of the tested product did not affect nutrient bioavailability, nor was this a contributing factor to its ineffectiveness. Likewise, the manufacturer’s claims do not support any detrimental effect of feed processing (i.e. steam pelleting) on the efficacy of the CFA. The potential influence of the complexity of the feed matrix on the functionality of the CFA may, however, be a topic of future study especially pertinent to the use of anti-mycotoxin agents in fish feeds.

To our knowledge, Hauptman et al. (2014) have conducted the only published study available to date on the efficacy of a feed additive in preventing the adverse effects of feed-borne Fusarium mycotoxins on fish. The effect of adding Mycofix® Plus (MFP), an additive with epoxidase, lactonase and esterase activities, to diets containing increasing levels (0, 15 and 30%) of Grain Distiller’s Dried Yeasts (GDDY) naturally contaminated with OTA, FB$_3$, fumonisin B$_3$ (FB$_3$), ZON and DON (0.9 ppb, 0.2 ppm, 0.1 ppm, 133 ppb and 0.7 ppm, respectively) was investigated in rainbow trout. The insignificant effects of MFP on growth performance and carcass proximate composition were in agreement with the effects of CFA in our study. However, an interactive effect of GDDY inclusion level and MFP was observed wherein protein retention efficiency (PRE) was increased by MFP supplementation in fish fed the diet containing 30% GDDY (estimated to contain 0.2 ppm DON). This is in contrast to our observation that inclusion of the CFA in diets predominantly contaminated with DON did not prevent decreased NRE. Diaz (2002) found that Mycofix® prevented the adverse effects of diets containing 1 ppm,
but not 2 ppm, of the trichothecene mycotoxin DAS on weight gain and FI of growing broilers, suggesting that the efficacy of some feed additives is potentially dependent on mycotoxin concentration(s). The 5- to 10-fold higher dietary concentrations of DON in our study compared to that of Hauptman et al. (2014) may explain the discrepancies between the efficacy of the CFA and MFP; still, a near significant, but non-specific, effect of MFP on ERE and a trend towards increased weight gain independent of dietary GDDY inclusion might alternatively indicate that the effect of MFP was not associated with mycotoxin contamination of the diets. The non-specific effects of anti-mycotoxin feed additives are an important consideration, particularly in the evaluation of an expanding number of novel feed ingredients such as ethanol yeasts for which dietary inclusion in fish feeds may be influenced by multiple factors.

4.6 – Conclusion

The increased use of economically sustainable plant protein sources in aquaculture feeds has significantly increased the risk of exposing farmed fish to mycotoxins. Feed additives including clay minerals, yeast cell wall-derived adsorbents and biotransforming agents based on microbial or enzymatic detoxification are widely used in various sectors of animal production to counteract the adverse effects of mycotoxin-contaminated feedstuffs; however, their application to prevent fusariotoxicoses in a number of commercially important fish species has been largely without scientific evidence. *In vitro* tests, although informative, may not always correlate well with *in vivo* effectiveness and should therefore not be used as the only indicator of efficacy (Diaz and Smith, 2005). This study represents a robust *in vivo* assessment of the efficacy of an unspecified CFA with adsorptive and microbial detoxifying capacity in minimizing...
or preventing the adverse effects of DON and co-occurring *Fusarium* mycotoxins on a number of parameters of practical importance in rainbow trout. Inclusion of the CFA at the recommended rate (2 g/kg feed) did not minimize the adverse effects of diets containing increasing, graded levels of DON (0.3, 1.0, 1.5 and 2.0 ppm) on growth performance, body composition or nutrient utilization of rainbow trout. The inefficacy of the CFA may be related to its use under physiological conditions which are outside the scope of its effectiveness (i.e. the gastrointestinal tract of a cold water fish species). Consequently, continued assessment of anti-mycotoxin solutions for fish and the systematic development of novel mitigation strategies are necessary.
CHAPTER 5 – A COMPARATIVE INVESTIGATION OF THE EFFECTS OF FEED-BORNE DEOXYNIVALENOL (DON) ON GROWTH PERFORMANCE, NUTRIENT UTILIZATION AND METABOLISM OF DETOXIFICATION IN RAINBOW TROUT (ONCORHYNCHUS MYKISS) AND NILE TILAPIA (OREOCHROMIS NILOTICUS) FED DIETS CONTAINING DIFFERENT LEVELS OF DIGESTIBLE CARBOHYDRATES

5.1 – Abstract

Increasing evidence suggests that variability in hepatic glucuronidation is a critical determinant of mammalian species-specific sensitivity to the Fusarium mycotoxin deoxynivalenol (DON). Concurrently, ongoing work regarding the effects of DON on fish has led to broad classifications concerning species-specific sensitivity (i.e. carnivorous species are highly sensitive to DON and omnivorous species are highly tolerant to DON). This study was designed to determine if sensitivity to DON in fish is related to inherent differences in UDP-glucuronosyltransferase (UDPGT) activity and to evaluate if increased dietary digestible starch content could be an effective strategy to increase glucuronidation capacity. Rainbow trout or Nile tilapia (initial average body weight=21.9 and 8.1 g/fish, respectively) were fed two series (12 or 24% digestible starch) of three diets containing graded levels of naturally occurring DON (0.1, 0.7 and 1.3 ppm) for 10 weeks. Significant linear decreases in weight gain, thermal-unit growth coefficient (TGC, P≤0.05), feed efficiency (P<0.001), whole body crude protein (CP) content (P<0.001), retained nitrogen (RN, P≤0.05) and nitrogen retention efficiency (NRE, P<0.01) were associated with increasing levels of DON in rainbow trout regardless of the dietary digestible starch content. There were no significant effects of DON on any of these parameters in Nile tilapia (P>0.05) with the exception of a quadratic increase in NRE (P<0.01) in fish fed the diets containing 24% digestible starch. The effect of DON on growth performance and nutrient utilization was not altered by the digestible starch content of the diet (P>0.05, DON × digestible...
starch). A significant quadratic decrease (P≤0.05) in UDPGT activity was associated with increasing levels of DON in rainbow trout fed the diet containing 12% digestible starch, whereas trout and tilapia fed the diet containing 24% digestible starch experienced significant quadratic or linear increases (P≤0.05) in UDPGT activity, respectively. However, UDPGT activity was approximately 10-fold higher in rainbow trout than in Nile tilapia. This suggests that UDPGT activity is not responsible for the observed species-specific sensitivity to DON or it is not an appropriate biomarker of DON glucuronidation. A significantly higher relative fold expression of CYP1A in tilapia compared to trout (P=0.0001) may offer potential opportunity for future study. We demonstrate for the first time that Nile tilapia are unaffected by practically relevant levels of DON which are detrimental to rainbow trout. This study also extends the current knowledge of DON metabolism and represents an important contribution to the development of efficacious nutritional mycotoxin mitigation strategies.

5.2 – Introduction

Deoxynivalenol (DON), a trichothecene mycotoxin produced mainly by *Fusarium graminearum* and *F. culmorum*, is a ubiquitous contaminant of small cereal grains (e.g. corn, wheat and barley) worldwide (Desjardins et al., 1993; Sudakin, 2003; Glenn, 2007; Streit et al., 2013; Schatzmayr and Streit, 2013). The thermal stability of DON renders it largely unaltered by routine food/feed processing techniques, thus making the potential of human and animal exposure a significant concern (Bennett and Richard, 1996; Hazel and Patel, 2004). Consumption of feedstuffs contaminated with DON results in a variety of adverse effects in animals. Prolonged feed-borne exposure to low doses of DON is commonly associated with
anorexia (reduced feed intake and growth), decreased productivity and altered nutritional efficiency. Comparatively, acute exposure to higher concentrations of DON is characterized by symptoms including leucocytosis, gastrointestinal hemorrhage, diarrhea and emesis or vomiting (notably in pigs; Forsyth et al., 1977; Young et al., 1983; Pestka et al., 1987; Rotter et al., 1996; Pestka, 2007). The yearly economic losses related to DON contamination of food crops, feed crops and livestock mortality in the United States alone have been estimated to be as high as $1.2 billion, $55 million and $5 million, respectively (CAST, 2003).

Substantial variability in sensitivity to DON exists between species. In general, the order of decreasing sensitivity to DON among the most frequently studied farm and experimental animals is: swine > rats > mice > poultry ≈ ruminants (Rotter et al., 1996). For example, reduced growth performance has been reported in young pigs fed diets containing 1 to 2 ppm DON, whereas short-term feeding of a ration containing 66 mg/kg DON did not affect feed intake or milk production in lactating dairy cows (Young et al., 1983; Côté et al., 1986). Rapid and efficient absorption and a limited or non-existent capacity to transform DON to its de-epoxy metabolite, DOM-1, via intestinal or rumen microbial activity in pigs compared to poultry and ruminants are widely cited as critical factors influencing the sensitivity of these species to DON (King et al., 1984; Prelusky et al., 1985, 1986a,b; 1987, 1988; Lun et al., 1988; He et al., 1992; Eriksen et al., 2003; Dänicke et al., 2004a,b). Concurrently, increasing evidence has indicated that glycosylation or glucuronidation of DON is extensively involved in its metabolism in plants, animals and humans and may be an important determinant of interspecies variability in sensitivity to DON (Meky et al., 2003; Poppenberger et al., 2003; Turner et al., 2011; Warth et al., 2012a, 2013; Maul et al., 2012).
Glucuronidation, a phase II metabolic reaction catalyzed by the uridine diphosphate glucuronosyltransferase (UDPGT) family of enzymes, involves the conjugation of a xenobiotic with uridine diphosphate glucuronic acid (UDPGA), generally resulting in the formation of a less toxic and water-soluble compound which can be more readily eliminated in bile, urine or feces (Tephly and Burchell, 1990; Tukey and Strassburg, 2000; Gibson and Skett, 2001; Brenner and Stevens, 2010). The availability of UDPGA appears to be a key rate-limiting factor for glucuronidation. That is, if UDPGA is depleted, a reduced rate of glucuronide formation will result (Reinke et al., 1981; Singh and Schwarz, 1981; Aw and Jones, 1984). UDP-glucuronate (anionic/physiologically active form of UDPGA) is produced as part of the uronic acid pathway from UDP-glucose, which, in turn, is derived from glucose 1-phosphate originating from either exogenous (dietary) glucose or glycogen through glycogenolysis (Reinke et al., 1994; Horton et al., 2002; Wamelink et al., 2008; Engelking, 2015; Bender, 2009b). The endogenous production of UDPGA as a co-factor in glucuronidation is therefore inherently connected to carbohydrate metabolism. Moreover, increasing the dietary carbohydrate to protein ratio has been shown to significantly increase the clearance rate and urinary recovery of glucuronide conjugates in human subjects administered drugs which are metabolized primarily by conjugation with UDPGA (Thurman and Kauffman, 1980; Sonne et al., 1989; Pantuck et al., 1991). This suggests that the rate of glucuronidation can be potentially influenced by the provision of dietary sources of glucose.

In addition to terrestrial mammals and birds, species-specific sensitivity to DON has been reported in fish. Generally, warm water omnivorous species are considered much more tolerant to DON-contaminated feeds than cold water carnivorous species (Spring and Fegan,
2005; Spring and Burel, 2008; Manning and Abbas, 2012). We previously observed highly significant linear or quadratic decreases in feed intake, weight gain, growth rate (expressed as thermal-unit growth coefficient, TGC) and feed efficiency in rainbow trout fed diets naturally contaminated with graded levels of DON ranging from 0.3 to 2.6 ppm (Hooft et al., 2011). In contrast, juvenile channel catfish were able to tolerate diets containing up to 10 ppm DON from a purified source or naturally contaminated wheat without any apparent adverse effects on growth performance (Manning et al., 2005). Interestingly, it has been established that omnivorous fish species (e.g. channel catfish) generally have a much greater ability to effectively utilize dietary carbohydrates and metabolize glucose compared to carnivorous species such as rainbow trout (Edwards et al., 1977; Furuichi and Yone, 1980; Hilton and Atkinson, 1982; Beamish et al., 1986; Hilton et al., 1987; Higgs et al., 1992; Mazur et al., 1992; Wilson, 1994). This has been effectively illustrated in glucose tolerance tests demonstrating that the duration of blood glucose elevation is significantly shorter in omnivorous species relative to carnivorous fish (Furuichi and Yone, 1981; Shiau and Chuang, 1995; Legate et al., 2001). Given the relationship between carbohydrate metabolism and glucuronidation, we hypothesized that the limited ability of some carnivorous fish species to metabolize glucose may result in a decreased rate of glucuronidation and thus, increased sensitivity to DON.

This study aimed to compare the effects of diets contaminated with practically relevant levels of DON on the growth performance and metabolism of a sensitive, carnivorous species, rainbow trout and an omnivorous species, Nile tilapia. More specifically, we sought to evaluate whether or not potential differences in sensitivity to DON between these species are related to differences in UDP-glucuronosyltransferase (UDPGT) activity associated with the greater
capacity of Nile tilapia to effectively utilize dietary carbohydrates relative to rainbow trout. To our knowledge, this is the first study to investigate the interaction of dietary composition and glucuronidation of DON. Accordingly, the work presented here may provide insight into nutritional mitigation strategies for counteracting the adverse physiological effects of DON.

5.3 – Materials and methods

5.3.1 – Fish husbandry, experimental conditions and sampling protocol

Rainbow trout with an initial average body weight of 21.9 g/fish and Nile tilapia with an initial average body weight of 8.1 g/fish were obtained from the Alma Aquaculture Research Station (Elora, ON, Canada) and Sand Plains AquaCulture (Mossley, ON, Canada), respectively. Fish were acclimated to the experimental conditions for two weeks prior to the start of the trial during which time both species were fed a maintenance ration of a high quality commercial trout feed (Martin Mills Inc., Elmira, Ontario, Canada).

Groups of 25 rainbow trout or Nile tilapia were randomly assigned to 36 fibreglass tanks (18 tanks/species), each with a volume of 60 L. Fish were maintained in a partial recirculation system (approximately 30% make up water) equipped with gravel biofilters (1 biofilter/9 tanks). Tanks were continuously and individually aerated and supplied with water at a rate of 3 L/min. Water temperature was maintained at 15.3 ± 0.4 °C for rainbow trout and 30.1 ± 0.8 °C for Nile tilapia by injection of hot water into the incoming water line using a solenoid valve controlled by a thermoregulator. Photoperiod was maintained at 12 hours light: 12 hours dark in a windowless laboratory. The animals were kept in accordance with the guidelines of the
Canadian Council on Animal Care (CCAC, 1984) and the University of Guelph Animal Care Committee.

Feed intake was recorded weekly and individual tanks of fish were weighed every 28 days over the course of the 10-week trial. A pooled sample of whole fish from each species was taken for determination of initial carcass composition. At the end of the trial, four fish per tank were randomly sampled for determination of final carcass composition. Fish sampled for analysis of proximate composition were stored at –20 °C until processing. Additionally, the livers of 12 fish per tank were sampled for analysis of gene expression using real-time PCR and UDP-glucuronosyltransferase activity. Briefly, livers were carefully excised to prevent rupture of the gallbladder, weighed, washed in phosphate-buffered saline (PBS), placed in 4 mL cryogenic tubes, immediately frozen in liquid nitrogen and stored at –80 °C until analysis. Fish were humanely euthanized by an overdose of tricaine methane sulfonate (200 mg/L water).

5.3.2 – Experimental diets and feeding protocol

Two sets of three experimental diets each (Diets 1-3 and Diets 4-6) were prepared to meet or exceed the nutrient requirements for both rainbow trout and Nile tilapia according to NRC (2011). Two sources of ground corn (< 2 mm), one known to be contaminated with Fusarium mycotoxins and one “clean” or relatively uncontaminated, were obtained from the Arkell Feed Mill (University of Guelph, Guelph, ON, Canada). Three levels of DON were attained by increasing the proportion of naturally contaminated corn to clean corn. Using this approach, Diets 1, 2 and 3 were formulated to have the same levels of DON as Diets 4, 5 and 6, respectively (Table 5.1). Within each set, diets were formulated to be isonitrogenous and
isoenergetic. Diets 1-3 were formulated to have a crude protein content of 40% and a digestible starch content of 12% (as is basis). In comparison, Diets 4-6 were formulated to contain 32% crude protein and 24% digestible starch. Total starch content of the two corn sources was determined using the Megazyme Total Starch Assay Kit according to the manufacturer’s protocol (Megazyme International Ireland, Wicklow, Ireland). The contribution of corn, gelatinized corn starch, corn protein concentrate and soybean meal to the crude protein and starch content of the diets was calculated and balanced to be the same within each set of diets. The digestible starch content of the diets was calculated using the model developed by Hua and Bureau (2009). Inclusion of pre-gelatinized corn starch in Diets 4-6 was accomplished by decreasing the content of highly digestible protein and lipid sources, mainly fish meal, poultry by-products meal and fish oil, relative to Diets 1-3 (Table 5.1). The levels of synthetic amino acids were increased accordingly in Diets 4-6 to ensure that the requirements were met for both species. Corn protein concentrate (CPC), soy protein concentrate (SPC) and corn oil within each set of diets were adjusted to account for differences in the analyzed crude protein and lipid composition of the clean and contaminated corn (Table 5.1).

Diets were mixed using a Hobart mixer (Hobart Ltd., Don Mills, ON, Canada), steam pelleted using a laboratory pellet mill (California Pellet Mill Co., San Francisco, CA, USA), dried under forced-air at room temperature for 24 hours, sieved and stored at 4 °C until used. Triplicate groups of each species were hand-fed the experimental diets to satiety three times daily on weekdays and once per day on weekends.
Table 5.1. Formulation of the experimental diets and calculated or analyzed composition.

<table>
<thead>
<tr>
<th>Ingredient (g/100 g diet)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal, herring</td>
<td>18.0</td>
<td>18.0</td>
<td>18.0</td>
<td>14.4</td>
<td>14.4</td>
<td>14.4</td>
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<tr>
<td>Poultry by-products meal</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>9.6</td>
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<tr>
<td>Feather meal</td>
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<td>4.0</td>
<td>4.0</td>
<td>3.2</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Corn, clean&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.56</td>
<td>10.78</td>
<td>-</td>
<td>21.56</td>
<td>10.78</td>
<td>-</td>
</tr>
<tr>
<td>Corn, contaminated&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>11.0</td>
<td>22.0</td>
<td>-</td>
<td>11.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Corn protein concentrate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.92</td>
<td>3.59</td>
<td>3.25</td>
<td>1.00</td>
<td>0.66</td>
<td>0.33</td>
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<tr>
<td>Soy protein concentrate 300&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.96</td>
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<td>4.80</td>
<td>4.77</td>
<td>4.75</td>
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<tr>
<td>Soybean meal</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Gelatinized corn starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Biolys®, 52 % lysine</td>
<td>1.38</td>
<td>1.39</td>
<td>1.40</td>
<td>2.21</td>
<td>2.22</td>
<td>2.23</td>
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<tr>
<td>DL-methionine</td>
<td>0.09</td>
<td>0.10</td>
<td>0.11</td>
<td>0.45</td>
<td>0.46</td>
<td>0.47</td>
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<tr>
<td>L-histidine</td>
<td>0.29</td>
<td>0.29</td>
<td>0.30</td>
<td>0.49</td>
<td>0.50</td>
<td>0.50</td>
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<tr>
<td>Choline chloride</td>
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<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
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<tr>
<td>Monocalcium phosphate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Ascorbic acid&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td>NaCl</td>
<td>0.3</td>
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<tr>
<td>Cellulose (Arbocel)</td>
<td>4.65</td>
<td>4.65</td>
<td>4.65</td>
<td>3.85</td>
<td>3.86</td>
<td>3.85</td>
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<tr>
<td>Vitamin premix&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.3</td>
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</tr>
<tr>
<td>Mineral premix&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.2</td>
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<td>0.2</td>
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<tr>
<td>Fish oil, herring</td>
<td>8.1</td>
<td>8.1</td>
<td>8.1</td>
<td>3.54</td>
<td>3.54</td>
<td>3.54</td>
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<tr>
<td>Corn oil</td>
<td>2.65</td>
<td>2.76</td>
<td>2.88</td>
<td>1.00</td>
<td>1.11</td>
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<tr>
<td>Total</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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</table>

**Calculated composition**

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<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross energy&lt;sup&gt;h&lt;/sup&gt; (kJ/g, dry matter basis)</td>
<td>22.2</td>
<td>22.2</td>
<td>22.2</td>
<td>20.4</td>
<td>20.4</td>
<td>20.4</td>
</tr>
<tr>
<td>Digestible starch content&lt;sup&gt;i&lt;/sup&gt; (% of diet)</td>
<td>12.2</td>
<td>12.2</td>
<td>12.2</td>
<td>24.0</td>
<td>24.0</td>
<td>24.0</td>
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</table>

**Analyzed composition (dry matter basis)**

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<tr>
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<tbody>
<tr>
<td>Dry matter (%)</td>
<td>94.6</td>
<td>94.5</td>
<td>94.7</td>
<td>92.4</td>
<td>92.5</td>
<td>92.2</td>
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<tr>
<td>Crude protein (%)</td>
<td>43.3</td>
<td>42.2</td>
<td>42.5</td>
<td>35.3</td>
<td>34.3</td>
<td>35.6</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>15.8</td>
<td>16.0</td>
<td>16.4</td>
<td>8.6</td>
<td>8.6</td>
<td>9.0</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.6</td>
<td>8.7</td>
<td>8.9</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analyzed composition: 8.9% crude protein, 3.3% lipid, 1.7% ash, 65.7% starch (dry matter basis).

<sup>b</sup> Analyzed composition: 12.2% crude protein, 1.8% lipid, 1.7% ash, 64.0% starch (dry matter basis).

<sup>c</sup> EmpyReal® 75 (Cargill Corn Milling, Minneapolis, MN, USA).

<sup>d</sup> HP 300 (Hamlet Protein, Horsens, Denmark).

<sup>e</sup> Rovimix® Stay-C® 25% (DSM, Heerlen, Netherlands).

<sup>f</sup> Provides per kg of diet: retinyl acetate (vitamin A), 75 mg; cholecalciferol (vitamin D3), 300 mg; menadione Na-bisulfate (vitamin K), 1.5 mg; cyanocobalamine (vitamin B12), 30 mg; D-biotin, 210 mg; choline chloride, 3448 mg; folic acid, 1.5 mg; niacin, 15 mg; calcium-d-pantothenate,
33 mg; pyridoxine–HCl, 7.5 mg; riboflavin, 9 mg; thiamin-HCl, 1.5 mg (Martin Mills Inc., Elmira, ON, Canada).

Provides per kg of diet: sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; potassium iodine (KI, 24%K, 76%I), 10.5 mg; ferrous sulphate (FeSO₄, 7H₂O, 20%Fe), 65 mg; manganese sulphate (MnSO₄, 36%Mn) 88.9 mg; zinc sulphate (ZnSO₄·7H₂O, 40%Zn), 150 mg; copper sulphate (CuSO₄·5H₂O, 25%Cu), 28 mg; sodium Selenite (Na₂SeO₃, 45.66% Se), 0.7 mg (Martin Mills Inc., Elmira, ON, Canada).

\[\text{[(Crude protein} \times 23.4 \text{ kJ/g}) + (\text{lipid} \times 39.2 \text{ kJ/g}) + (\text{NFE} \times 17.2 \text{ kJ/g})] \div \% \text{ dry matter (Cho et al., 1982)}\]

Calculated according to Hua and Bureau (2009).
5.3.3 – Mycotoxin analysis

Representative samples of the two sources of corn and six experimental diets were analyzed for multiple fungal metabolites (63 in corn and 47 in the diets) and two bacterial metabolites according to the method of Malachová et al. (2014) using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Department for Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences, Tulln, Austria). Briefly, 5 g of each sample was extracted for 90 min with 20 mL acetonitrile/water/acetic acid (79:20:1, v/v/v) on a rotary shaker (GFL 3017, GFL, Burgwedel, Germany). The crude extracts were diluted 1+1 (v+v) with acetonitrile/water/acetic acid (20:79:1, v/v/v) and 5 µL of the diluted extract was injected. Detection and quantification were performed with a LC-MS/MS system (QTrap5500, Applied Biosystems, Foster City, CA) equipped with an electrospray ionisation (ESI) source (TurbolonSpray) and an ultra-high performance liquid chromatography system (1290 Series, Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini C18-column (150 × 4.6 mm inner diameter, 5 µm particle size), equipped with a C18 security guard cartridge (4 × 3 mm inner diameter; all from Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid in a ratio of 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B). After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 µL/min. Data acquisition was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative
polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the retention time: ± 27 s and ± 42 s in positive and negative modes, respectively. The target scan time was set to 1 s. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid that each exhibit only one fragment ion). Analyst® software (version 1.5.1, AB Sciex, Foster City, CA, USA) was used to control the LC-MS/MS instrument, as well as for automatic and manual integration of the peak. Quantification of the > 300 metabolites included in the method was done based on linear, 1/x weighed calibration curves derived from the analysis of serial dilutions of a multi-analyte stock solution. Results were not corrected for apparent recoveries due to lack of a suitable blank sample. The accuracy of the method is verified on a routine basis by participation in a proficiency testing scheme organized by BIPEA (Gennevilliers, France), which includes samples of animal feed.

5.3.4 – Proximate analysis and gross energy calculation

Diet, ingredient (≥ 3% of the diet) and carcass samples were analyzed for dry matter (DM) and ash according to AOAC (1995), crude protein (CP, %N × 6.25) by the Kjeldahl method using a Tecator™ Digestor for digestion with sulfuric acid and a Kjeltec 8200 for semi-automated distillation (Foss, Hillerød, Denmark), and lipids with an Ankom XT20 fat analyzer (Ankom Technology, Macedon, NY, USA) using petroleum ether. In preparation for analysis, samples of the pelleted diets and raw ingredients were ground to a fine, homogenous consistency using a laboratory scale grinder. Fish previously sampled and frozen for analysis of carcass composition were thawed overnight at room temperature, cooked in an autoclave,
ground into a homogenous slurry using a food processor, freeze dried, reground and stored at –20 °C until proximate analysis. Gross energy (GE; kJ/g) content of the diet and carcass samples was calculated according to Cho et al. (1982): \[
\frac{(CP \times 23.4 \text{ kJ/g}) + (\text{lipid} \times 39.2 \text{ kJ/g}) + (\text{NFE} \times 17.2 \text{ kJ/g})}{\% \text{ dry matter}}
\]
where: \(CP = \% \text{ crude protein (as is basis, } \% N \times 6.25)\); \(\text{lipid} = \% \text{ lipid (as is basis)}\); \(\text{NFE} = \% \text{ nitrogen-free extract (as is basis)}\).

**5.3.5 – Growth performance and nutrient utilization calculations**

Growth rate, expressed as thermal-unit growth coefficient (TGC), was calculated for each tank as: \(TGC = 100 \times (\text{FBW}^{1/3} - \text{IBW}^{1/3}) \times (\text{sum } T \times D)^{-1}\), where: \(\text{FBW} = \text{final body weight (g/fish)}\); \(\text{IBW} = \text{initial body weight (g/fish)}\); and \(\text{sum } T \times D = \text{sum degrees Celsius } \times \text{ days}\).

Feed efficiency (FE, gain:feed) was calculated for each tank as: \(\text{FE} = \frac{\text{live body weight gain}}{\text{dry feed intake}}\), where: \(\text{feed intake} = \frac{\text{total dry feed}}{\text{number of fish}}\); and \(\text{live body weight gain} = \frac{\text{FBW/initial number of fish}}{\text{IBW/initial number of fish}}\).

Retained nitrogen (RN, g/fish) and recovered energy (RE, KJ/fish) were calculated for each tank as: \(\text{RN} = (\text{FBW} \times \text{N content}_{\text{final}}) - (\text{IBW} \times \text{N content}_{\text{initial}})\) and \(\text{RE} = (\text{FBW} \times \text{GE content}_{\text{final}}) - (\text{IBW} \times \text{GE content}_{\text{final}})\), respectively, where: \(\text{N content}_{\text{final}} = \text{nitrogen content (g/fish)}\) of the final carcass sample; \(\text{N content}_{\text{initial}} = \text{nitrogen content (g/fish)}\) of the initial carcass sample; \(\text{GE}_{\text{final}} = \text{gross energy (kJ/g) content of the final carcass sample}\); and \(\text{GE}_{\text{initial}} = \text{gross energy (kJ/g) content of the initial carcass sample}\).

Nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were calculated for each tank as a percentage of ingested nitrogen (IN, g/fish) and ingested energy.
(IE, KJ/fish), respectively: NRE (% IN)=\[
\frac{([FBW \times N \text{ content}_{\text{final}}] - [IBW \times N \text{ content}_{\text{initial}}])}{IN}\] \times 100

and ERE (% IE)=\[
\frac{([FBW \times GE \text{ content}_{\text{final}}] - [IBW \times GE \text{ content}_{\text{initial}}])}{IE}\] \times 100.

5.3.6 – RNA extraction and real-time PCR

Total RNA was isolated from 30 mg of liver tissue pooled from three fish per tank (3 replicates per experimental treatment) using the RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. Samples were treated to eliminate possible genomic DNA contamination using the RNase-Free DNase Set (Qiagen GmbH, Hilden, Germany). RNA quality and integrity was assessed by agarose gel electrophoresis using ethidium bromide staining to qualitatively verify the presence of sharp, clear 28S and 18S rRNA bands in an approximate ratio of 2:1. RNA quantity and purity were determined by spectrophotometry at 260 and 280 nm (Biochrom Ultrospec 2000, Biochrom Ltd., Cambridge, England). First-strand cDNA was synthesized from total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using random primers according to the manufacturer’s instructions.

Real-time PCR was performed to determine the relative expression of selected genes. Primers were synthesized by Laboratory Services (University of Guelph, ON, Canada) and are presented in Table 5.2. Real-time PCR amplifications were carried out in triplicate (Rotor-Gene™ 3000, Corbett Research, Sydney, Australia) in strip tubes with a total reaction volume of 20 µL containing 2 µL diluted (1/10) cDNA template, 1.2 µL of forward and reverse primer (2 mmol/L), 10 µL of 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 5.6 µL DEPC water. No template controls (NTCs) were included in each experiment.
Thermal cycling was initiated with an activation step at 95 °C for 15 min, followed by 45 cycles of amplification comprised of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Following the final cycle, amplification of a single product was verified by observation of a single peak at the expected Tm on the melting curve. Amplification efficiencies were determined by generation of a standard curve with serial dilutions of pooled cDNA. Relative quantification of the target gene transcript was calculated according to the Pfaffl method (Pfaffl, 2001), which determines an expression ratio based on real-time PCR efficiency and the crossing point deviation of an unknown sample versus a control:

\[
\frac{\left( E_{\text{target}} \Delta CT_{\text{target(control-sample)}} \right)}{\left( E_{\text{ref}} \Delta CT_{\text{ref(control-sample)}} \right)}
\]

where: \( E_{\text{target}} \) is the real-time PCR efficiency of the target gene transcript; \( E_{\text{ref}} \) is the real-time PCR efficiency of the reference gene transcript (β-actin); \( \Delta CT_{\text{target}} \) is the difference in the Ct values of the control (Diet 1) and sample of the target gene transcript; and \( \Delta CT_{\text{ref}} \) is the difference in the Ct values of the control (Diet 1) and sample of the reference gene (β-actin) transcript.
<table>
<thead>
<tr>
<th>Species</th>
<th>Transcript</th>
<th>Primer sequence (5’-3’)</th>
<th>Fragment (bp)</th>
<th>Accession No.</th>
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<tr>
<td>OM</td>
<td>β-actin</td>
<td>F: TGTGACGTGGACATCCGTAA R: TGCTGGAAGGTGGAGAGAGA</td>
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<td>OM</td>
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\(^a\) *Oncorhynchus mykiss* (rainbow trout)
\(^b\) *Oreochromis niloticus* (Nile tilapia)
\(^c\) GenBank (http://www.ncbi.nlm.nih.gov/)
\(^d\) Ensembl (http://www.ensembl.org/index.html)
5.3.7 – UDP-glucuronosyltransferase activity

UDP-glucuronosyltransferase (UDPGT) activity was determined according to the development of a microplate method based on the work of Cástren and Oikari (1983). Briefly, individual livers from four fish per tank (approximately 1 g where possible) were homogenized using a Polytron homogenizer (Kinematica AG, Switzerland) in 4 vol of 0.25 M sucrose on ice and centrifuged at 12,000 × g for 20 min. The supernatant was collected and ultracentrifuged at 100,000 × g for 60 min (Sorvall™ WX Ultra Series, Thermo Fisher Scientific Inc., MA, USA). Following ultracentrifugation, the resulting supernatant was carefully discarded and the pellet was resuspended in 1 mL of 0.25 M sucrose. Microsomal preparations were stored at −80 °C until analysis of UDPGT activity. A small aliquot of each sample (< 100 µL) was used for determination of protein concentration using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) was stored separately to prevent unnecessary thawing.

UDPGT activity was determined using p-nitrophenol as an aglycone and uridine 5′-diphosphoglucuronic acid ammonium salt (UDPGA) as the glucuronyl donor. Stock solutions of 0.5 M K₂EDTA (ethylenediaminetetraacetic acid dipotassium salt dehydrate) and 60 mM p-nitrophenol were prepared with 0.5 M potassium phosphate buffer (pH 7) in advance and stored at 4 °C. Immediately prior to conducting the assay, microsomal preparations were thawed and diluted to a final protein concentration of 1 mg/mL for rainbow trout and 2 mg/mL for Nile tilapia using 0.25 M sucrose and stored on ice until use. Potassium phosphate buffer (0.5 M, pH 7) containing 10 mM K₂EDTA was used to prepare an appropriate volume of dilution buffer containing 6.89 mM UDPGA. A solution containing 0.6 mM p-nitrophenol was then prepared using this dilution buffer. Samples were assayed in triplicate in 96-well plates. Each
well contained 100 µL diluted microsome to which 100 µL of the p-nitrophenol solution (containing 0.6 mM p-nitrophenol, 10 mM K₂EDTA and 6.89 mM UDPGA) was added. For each sample, blanks consisting of 100 µL diluted microsome and 100 µL dilution buffer were included on the same plate (also in triplicate). Temperature was maintained at 25 °C and absorbance was read at 400 nm every 5 min over a 90 min period using a BioTek PowerWave XS microplate spectrophotometer equipped with KC4 software (BioTek, Winooski, VT, USA). Each sample reading was adjusted by subtraction of the average absorbance of the respective blank. The amount of p-nitrophenol remaining at each time point was determined using a standard curve generated by serial dilution of a p-nitrophenol solution (containing 10 mM K₂EDTA and 6.89 mM UDPGA). Subsequently, the amount of p-nitrophenol glucuronide present at each interval was determined by difference relative to the amount of p-nitrophenol present at the initial reading \( p\text{-nitrophenol}_{\text{initial}} - p\text{-nitrophenol}_x \), where \( p\text{-nitrophenol}_{\text{initial}} \) is the amount of p-nitrophenol in nmol at the initial reading and \( p\text{-nitrophenol}_x \) is the amount of p-nitrophenol in nmol at a later time, \( x \). The amount of p-nitrophenol versus time was plotted for each sample to ensure that specific activity (nmol/min/mg protein) was calculated over a linear range (Figure 5.1). Total activity (nmol/min) was determined as follows: specific activity (nmol/min/mg) \( \times \) [protein (mg/mL) \( \div \) sample weight (mg)] \( \times \) liver weight (mg), where protein is the protein concentration in mg/mL of the homogenized sample; sample weight is the amount of liver homogenized in mg; and liver weight is the total liver weight in mg.
5.3.8 – Statistical analysis

All growth performance, nutrient utilization and gene expression data were analyzed as a 3 × 2 × 2 randomized incomplete block design using the PROC MIXED procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, USA), in which DON, species and starch were the treatment factors. All possible interaction terms were included in the model, and block was included as a random effect. Tank was considered the experimental unit. The Shapiro-Wilk test in PROC UNIVARIATE of SAS was used to assess normality, and the Brown and Forsythe test in PROC GLM of SAS was used to test for homogeneity of variances for all dependent variables prior to other statistical analysis. Variable transformation was adopted as necessary and heterogeneity of variances was accounted for in the model if required. Relative fold changes in gene expression were log transformed. Linear and quadratic orthogonal polynomial contrasts across levels of DON within each level of digestible starch content of the diet (for each species) were performed for all dependent variables. Contrast coefficients for equally spaced levels of DON (0.1, 0.7 and 1.3 ppm) were used (Kuehl, 2000). Data for UDP-glucuronosyltransferase activity was analyzed separately for each species as a 3 × 2 randomized incomplete block design with DON and starch as the treatment factors. An interaction term (DON × starch) was included in the model. Other statistical analysis was performed for each species as described above. Significance was declared at P≤0.05.
Figure 5.1. Examples of the graphical representation of the formation of p-nitrophenol (p-NP) glucuronide over time by liver microsomes obtained from one tank of rainbow trout (A) and one tank of Nile tilapia (B) fed Diet 3. Data points are means of 3 replicates per fish ± SEM.
5.4 – Results

5.4.1 – Mycotoxin concentrations

In addition to DON, the following fungal metabolites were positively identified in the clean and/or contaminated corn and in one or more of the diet samples: DON-3-glucoside, zearalenone, zearalenone-4-sulfate, α-zearalenol, β-zearalenol, T-2 toxin, HT-2 toxin, sterigmatocystin, averufin, averufanin, norsolorinic acid, versicolorin C, nidurufin, aspterric acid, skyrin, beauvericin, enniatin B, enniatin B1, enniatin A, enniatin A1, aurofusarin, culmorin, 15-hydroxyculmorin, 5-hydroxyculmorin, 15-hydroxyculmoron, equisetin, moniliformin, monocerin, alternariol, alternariolmethyl ether, tenuazonic acid, tentoxin, cyclosporin A, cyclosporin C, cyclosporin H, tryptophol, brevinamid F and emodin. Other metabolites including nivalenol, griseofulvin, macrosporin, cytochalasin D, cytochalasin J and physcion were found at detectable levels in one or more of the diet samples. Metabolites analyzed only in the corn samples and present at detectable levels in the clean and/or contaminated corn include: fumonisin B1, fumonisin B2, ochratoxin A, averantin, versicolorin A, 3-nitropropionic acid, terphenyllin, cycloaspeptide A, fusaproliferin, bikaverin, butenolid, apicidin, fusaric acid, fusarimonic acid, sambucinol, cyclosporin D, chanoclavine, festuclavin, viridicatin, methylviridicatin and curvularin.

The clean corn contained 0.649 ppm DON, while the contaminated corn was found to contain 5.198 ppm DON. Samples of Diets 1, 2, 3, 4, 5 and 6 had DON concentrations of 0.148, 0.738, 1.246, 0.127, 0.750 and 1.359 ppm, respectively. For statistical analysis, average DON concentrations of 0.1, 0.7 and 1.3 ppm were used. The concentrations of DON in the corn and diet samples and other mycotoxins detected at levels within this range (i.e. above 0.1 ppm) are
presented in Table 5.3. Notably, aurofusarin was detected in the diet samples at concentrations similar to or exceeding those of DON. Likewise, the diets contained graded levels of 15-hydroxyculmorin and 5-hydroxyculmorin ranging from 0.071 to 1.090 ppm and 0 (< LOD) to 1.256 ppm, respectively.
Table 5.3. Occurrence of fungal metabolites present in the corn sources and experimental diets determined by LC-MS/MS.

<table>
<thead>
<tr>
<th>Metabolite (ppm)</th>
<th>Corn source</th>
<th>Experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean</td>
<td>Cont.(^a)</td>
</tr>
<tr>
<td>DON</td>
<td>0.649</td>
<td>5.198</td>
</tr>
<tr>
<td>DON-3-glucoside</td>
<td>0.122</td>
<td>1.101</td>
</tr>
<tr>
<td>ZON</td>
<td>0.021</td>
<td>0.992</td>
</tr>
<tr>
<td>Cycloaspeptide A</td>
<td>&lt; LOD(^b)</td>
<td>0.122</td>
</tr>
<tr>
<td>Aurofusarin</td>
<td>0.538</td>
<td>7.126</td>
</tr>
<tr>
<td>Culmorin</td>
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<td>0.985</td>
</tr>
<tr>
<td>15-hydroxyculmorin</td>
<td>0.618</td>
<td>5.888</td>
</tr>
<tr>
<td>5-hydroxyculmorin</td>
<td>0.269</td>
<td>4.526</td>
</tr>
<tr>
<td>15-hydroxyculmoron</td>
<td>0.129</td>
<td>1.916</td>
</tr>
<tr>
<td>Fusaric acid</td>
<td>0.046</td>
<td>0.405</td>
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<tr>
<td>Fusarinolic acid</td>
<td>0.058</td>
<td>0.314</td>
</tr>
<tr>
<td>Cyclosporin A</td>
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<tr>
<td>Cyclosporin C</td>
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<tr>
<td>Cyclosporin H</td>
<td>&lt; LOD</td>
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</tr>
<tr>
<td>Tryptophol</td>
<td>&lt; LOD</td>
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</tr>
<tr>
<td>Brevinamid F</td>
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<td>0.055</td>
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</table>

\(^a\)Contaminated corn.
\(^b\)Below the limit of detection (LOD).
\(^c\)Not applicable (metabolite not analyzed).
5.4.2 – Growth performance

The growth curves of rainbow trout and Nile tilapia fed the experimental diets are shown in Figures 5.2 and 5.3, respectively. Overall, dietary DON level had a significant effect on TGC (P<0.05) and feed efficiency (P<0.0001), while weight gain (P<0.01), TGC (P<0.01) and feed efficiency (P<0.0001) were significantly affected by digestible starch content. Rainbow trout had significantly higher growth rate (TGC) compared to Nile tilapia (P<0.0001). Conversely, Nile tilapia had significantly better feed efficiency than rainbow trout (P<0.05). A significant interaction of DON and species was observed for weight gain (P<0.01), TGC (P<0.01) and feed efficiency (P<0.001), indicating that rainbow trout and Nile tilapia responded differently to the contaminated feeds. A statistically significant interaction of starch and species was found for feed intake (P<0.05) and feed efficiency (P<0.01). There was no significant interaction of DON and starch for any of the growth performance parameters investigated suggesting that the response to DON was independent of dietary starch content. However, a trend toward a significant three-way interaction of DON, starch and species (P<0.07) with regard to TGC was noted (Table 5.4).

Significant linear decreases in weight gain (P<0.01), TGC (P<0.001), feed intake (P<0.05) and feed efficiency (P<0.0001) of rainbow trout were associated with increasing, graded levels of DON in diets formulated to contain 40% crude protein and 12% digestible starch. Likewise, significant linear decreases in weight gain (P<0.05), TGC (P<0.05) and feed efficiency (P<0.001) were also observed in rainbow trout fed diets containing graded levels of DON, but formulated to a higher digestible starch content (24%) (Table 5.4; Figures 5.4 A and 5.5 A). No significant linear or quadratic effects were associated with feeding Nile tilapia either set of diets (12 or
24% digestible starch) (Table 5.4; Figures 5.4 B and 5.5 B). However, trends toward significant increases in weight gain and feed intake (P<0.07) of Nile tilapia were associated with increasing concentrations of DON present in the diets containing 40% crude protein and 12% digestible starch. Mortality (data not shown) was not significantly affected by the experimental diets in either species.
Figure 5.2. Growth curves of rainbow trout (IBW=21.9 g/fish) fed diets with graded levels of DON formulated to contain 40% crude protein and 12% digestible starch (A) or 32% crude protein and 24% digestible starch (B).
Figure 5.3. Growth curves of Nile tilapia (IBW=8.1 g/fish) fed diets with graded levels of DON formulated to contain 40% crude protein and 12% digestible starch (A) or 32% crude protein and 24% digestible starch (B).
Table 5.4. Weight gain, thermal-unit growth coefficient, feed intake and feed efficiency of rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days.

<table>
<thead>
<tr>
<th>DON (ppm)</th>
<th>Digestible starch (%) as is</th>
<th>Gain (g/fish)</th>
<th>TGC(^a)</th>
<th>Feed intake (g/fish)</th>
<th>FE(^b) (gain/feed)</th>
</tr>
</thead>
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<tr>
<td><strong>Rainbow trout</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>12</td>
<td>89.9</td>
<td>0.198</td>
<td>96.3</td>
<td>0.93</td>
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<td>0.7</td>
<td>12</td>
<td>83.7</td>
<td>0.180</td>
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<td>***</td>
<td>*</td>
<td>****</td>
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<tr>
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<td>N.S.</td>
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</tr>
<tr>
<td><strong>Nile tilapia</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
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<td>0.175</td>
<td>108.6</td>
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<td>*</td>
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<tr>
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<tr>
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<td>**</td>
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<td>****</td>
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<tr>
<td>Species</td>
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<td>****</td>
<td>N.S.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>DON × Starch</td>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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</tr>
<tr>
<td>DON × Species</td>
<td>**</td>
<td>**</td>
<td>¥</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Starch × Species</td>
<td>N.S.</td>
<td>N.S.</td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>DON × Starch × Species</td>
<td>N.S.</td>
<td>¥</td>
<td>N.S.</td>
<td>N.S.</td>
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<tr>
<td>S.E.M.(^h)</td>
<td>8.3</td>
<td>N/A(^i)</td>
<td>8.6</td>
<td>0.02</td>
<td></td>
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</table>

\(^a\) TGC: Thermal-unit growth coefficient
\(^b\) FE: Feed efficiency
\(^a\) TGC = thermal-unit growth coefficient  
\(^b\) FE = feed efficiency  
\(^c\) Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to rainbow trout.  
\(^d\) Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to rainbow trout.  
\(^e\) Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to Nile tilapia.  
\(^f\) Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to Nile tilapia.  
\(^g\) Significance of the main (fixed) effects and interactions.  
\(^h\) S.E.M. = standard error mean.  
\(^i\) Pooled standard error of the mean not applicable (separate variance structure fit for statistical analysis of dependent variable).  
\(^j\) N.S. = not statistically significant.  
$\boldsymbol{\gamma} \; P \leq 0.07; \; * \; P \leq 0.05; \; ** \; P \leq 0.01; \; *** \; P \leq 0.001; \; **** \; P \leq 0.0001$
Figure 5.4. Thermal-unit growth coefficient (TGC) of rainbow trout (A, IBW=21.9 g/fish) and Nile tilapia (B, IBW=8.1 g/fish) fed diets with graded levels of DON containing 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (▲).
Figure 5.5. Feed efficiency (gain/feed) of rainbow trout (A, IBW=21.9 g/fish) and Nile tilapia (B, IBW=8.1 g/fish) fed diets with graded levels of DON containing 40% crude protein and 12% digestible starch.
5.4.3 – Carcass composition and nutrient utilization efficiency

The effects of the experimental diets on carcass composition and nutrient utilization are presented in Tables 5.5 and 5.6, respectively. Whole body crude protein content was significantly affected by DON (P<0.001), while dietary digestible starch content had a significant effect on carcass water (P<0.01), lipid (P<0.01) and calculated gross energy content (P<0.01). Nile tilapia had significantly lower whole body water content (P<0.05), but higher gross energy (P<0.001) content relative to rainbow trout. A significant interaction of DON and species (P<0.001) was observed for carcass crude protein composition. Crude protein content decreased linearly (P<0.001) with increasing levels of DON in rainbow trout fed either the high protein, low digestible starch or the low protein, high digestible starch diets (Figure 5.6 A). Additionally, a significant linear increase (P<0.05) in carcass lipid content of rainbow trout was associated with graded levels of DON in the diets formulated to contain 32% crude protein and 24% digestible starch. Comparatively, no significant linear or quadratic effects of graded levels of DON on whole body crude protein or lipid content of Nile tilapia were observed (Table 5.5; Figure 5.6 B). However, there was a significant linear increase in ash content (P<0.05) of Nile tilapia fed the diets formulated to contain 24% digestible starch. Only carcass ash content was significantly (P<0.05) affected by the three-way interaction of the independent variables.

RN (P<0.05) and NRE (P<0.001) were significantly affected by dietary DON concentration, whereas digestible starch content had a significant effect on RN (P<0.01), RE (P<0.01) and ERE (P<0.0001). In terms of species differences, Nile tilapia had significantly higher ERE (P<0.0001) than rainbow trout. Despite no significant difference between species with regard to carcass crude protein content, NRE of Nile tilapia was significantly higher
(P<0.05) compared to that of rainbow trout. Nutrient retention and utilization differed between species in response to DON, as indicated by the significant interaction of DON and species for RE (P<0.05), NRE (P<0.0001) and ERE (P<0.05). A significant interaction of starch and species was noted for NRE (P<0.01); however, RE, RN and nutrient utilization efficiencies were not significantly affected by the interaction of DON and starch. Significant linear decreases in RN (P<0.01), RE (P<0.05), NRE (P<0.01) and ERE (P<0.01) of rainbow trout fed the diets containing 12% digestible starch were associated with increasing levels of DON; concurrently, their counterparts fed the diets containing 24% digestible starch experienced significant linear reductions in RN (P<0.05) and NRE (P<0.01) only (Table 5.6; Figures 5.7 A and 5.8 A). In contrast to rainbow trout, feeding Nile tilapia the diets containing 12 or 24% digestible starch with graded levels of DON resulted in a significant linear increase in RE (P<0.05) and a significant quadratic response of NRE (P<0.01), respectively (Table 5.6, Figure 5.7 B). No significant linear or quadratic effects on ERE (P>0.05) were associated with feeding Nile tilapia diets containing graded levels of DON regardless of the formulated digestible starch content (Figure 5.8 B).
Table 5.5. Chemical body composition of the whole carcass of rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days.

<table>
<thead>
<tr>
<th>DON (ppm)</th>
<th>Digestible starch (% as is)</th>
<th>H₂O (%)</th>
<th>CP&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>GE&lt;sup&gt;b&lt;/sup&gt; (KJ/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>12</td>
<td>71.5</td>
<td>16.7</td>
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<tr>
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<td>12</td>
<td>71.4</td>
<td>16.2</td>
<td>9.8</td>
<td>2.1</td>
<td>7.7</td>
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<tr>
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<td>12</td>
<td>71.8</td>
<td>15.5</td>
<td>9.8</td>
<td>2.2</td>
<td>7.6</td>
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<td><strong>Significance</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>72.4</td>
<td>16.2</td>
<td>8.5</td>
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</tr>
<tr>
<td>Linear</td>
<td>N.S.</td>
<td>***</td>
<td>*</td>
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<td>N.S.</td>
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</tr>
<tr>
<td>DON</td>
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<td>***</td>
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<tr>
<td>Starch</td>
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<td>N.S.</td>
<td>**</td>
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</tr>
<tr>
<td>Species</td>
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<td>N.S.</td>
<td>¥</td>
<td>***</td>
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</tr>
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<td>N.S.</td>
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</tr>
<tr>
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<td>N.S.</td>
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</tr>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>DON × Starch × Species</td>
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<td>N.S.</td>
<td>N.S.</td>
<td>*</td>
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<td>S.E.M.&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>0.3</td>
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</table>

<sup>a</sup>CP=crude protein

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Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to rainbow trout.

Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to rainbow trout.

Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to Nile tilapia.

Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to Nile tilapia.

Significance of the main (fixed) effects and interactions.

S.E.M. = standard error mean.

N.S. = not statistically significant.

¥ P ≤ 0.07; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001
Figure 5.6. Whole body crude protein (CP, %) content of rainbow trout (A, IBW=21.9 g/fish) and Nile tilapia (B, 8.1 g/fish) fed diets with graded levels of DON containing 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (▲).
Table 5.6. Retained nitrogen, recovered energy, nitrogen retention efficiency and energy retention efficiency of rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days.

<table>
<thead>
<tr>
<th>DON (ppm)</th>
<th>Digestible starch (% as is)</th>
<th>RN$^a$ (g/fish)</th>
<th>RE$^b$ (KJ/fish)</th>
<th>NRE$^c$ (% IN)</th>
<th>ERE$^d$ (% IE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rainbow trout</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>36.2</td>
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<td></td>
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</tr>
<tr>
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<td>*</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
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<tr>
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<td>N.S.</td>
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</tr>
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<td></td>
</tr>
<tr>
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<td>*</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td><strong>Starch</strong></td>
<td>**</td>
<td>**</td>
<td>N.S.</td>
<td>****</td>
<td></td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td>N.S.</td>
<td>¥</td>
<td>*</td>
<td>****</td>
<td></td>
</tr>
<tr>
<td><strong>DON × Starch</strong></td>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td><strong>DON × Species</strong></td>
<td>N.S.</td>
<td>¥</td>
<td>*</td>
<td>****</td>
<td></td>
</tr>
<tr>
<td><strong>Starch × Species</strong></td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td><strong>DON × Starch × Species</strong></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>S.E.M.$^j$</td>
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<td>0.9</td>
<td>N/A$^k$</td>
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</tbody>
</table>

$^a$RN=retained nitrogen.
**b**RE=recovered energy.
**c**NRE (% IN)=nitrogen retention efficiency (% ingested nitrogen).
**d**ERE (% IE)=energy retention efficiency (% ingested energy).
**e**Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to rainbow trout.
**f**Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to rainbow trout.
**g**Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 12% digestible starch and graded levels of DON fed to Nile tilapia.
**h**Significance of the linear and quadratic orthogonal polynomial contrasts of dependent variables across experimental diets formulated to contain 24% digestible starch and graded levels of DON fed to Nile tilapia.
**i**Significance of the main (fixed) effects and interactions.
**j**S.E.M.=standard error mean.
**k**Pooled standard error of the mean not applicable (separate variance structure fit for statistical analysis of dependent variable).
**l**N.S.=not statistically significant.
¥ $P \leq 0.07$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$
Figure 5.7. Nitrogen retention efficiency (NRE) as a percentage of ingested nitrogen (% IN) of rainbow trout (A) or Nile tilapia (B) fed diets with graded levels of DON formulated to contain 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (▲).
Figure 5.8. Energy retention efficiency (ERE) as a percentage of ingested energy (% IE) of rainbow trout (A) or Nile tilapia (B) fed diets with graded levels of DON formulated to contain 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (▲).
5.4.4 – Gene expression

The relative expression of CYP1A was significantly affected by starch (P<0.02), species (P=0.0001) and the interaction of starch and species (P=0.001) (Table 5.7). No significant linear or quadratic effects of graded levels of DON on the expression of CYP1A in fish fed the diets containing either the low or high content of digestible starch were observed (Figure 5.9). The expression of UGT1A was not significantly affected by DON, starch or species or their interactions; however, the expression of UGT1A was linearly up-regulated (P<0.01) in Nile tilapia fed the diets containing increasing, graded levels of DON and 24% digestible starch (Table 5.7; Figure 5.10).

5.4.5 – UDP-glucuronosyltransferase activity

Total hepatic UDPGT activity (nmol/min/fish) of rainbow trout was significantly affected by DON (P<0.05), dietary digestible starch content (P<0.01) and the interaction of DON and starch (P<0.05) (Table 5.8). A significant quadratic decrease (P<0.05) in UDPGT activity was observed in rainbow trout fed the diets formulated to contain 40% crude protein and 12% digestible starch as the concentration of DON increased. Conversely, graded levels of DON in the diets containing 32% crude protein and 24% digestible starch resulted in a significant quadratic increase (P<0.05) in total UDPGT activity of rainbow trout (Figure 5.11).

No significant effects of DON or starch (P>0.05) on UDPGT activity of Nile tilapia fed the experimental diets were noted; however, a significant interaction of DON and starch (P<0.05) was found (Table 5.8). In agreement with the findings in rainbow trout, total hepatic UDPGT activity of Nile tilapia increased linearly (P<0.05) as the level of DON increased in the diets.
containing 24% digestible starch. In contrast, there were no significant linear or quadratic effects ($P > 0.05$) of graded levels of feed-borne DON on hepatic UDPGT activity of Nile tilapia fed the diets containing 12% digestible starch (Figure 5.12).
Table 5.7. Significance of the fixed effects for relative fold change in transcript expression of CYP1A and UGT1A in rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days.

<table>
<thead>
<tr>
<th>Effects</th>
<th>CYP1A</th>
<th>UGT1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>N.S.(^a)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Starch</td>
<td>P&lt;0.02</td>
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</tr>
<tr>
<td>Species</td>
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</tr>
<tr>
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<td>N.S.</td>
</tr>
<tr>
<td>DON × Species</td>
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<td>N.S.</td>
</tr>
<tr>
<td>Starch × Species</td>
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</tr>
<tr>
<td>DON × Starch × Species</td>
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<td>N.S.</td>
</tr>
</tbody>
</table>

\(^a\)N.S.=not statistically significant (P>0.05).
Table 5.8. Significance of the fixed effects for total hepatic UDPGT activity of rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight =8.1 g/fish) fed the experimental diets for 70 days.

<table>
<thead>
<tr>
<th>Effects</th>
<th>Rainbow trout</th>
<th>Nile tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>P&lt;0.05</td>
<td>N.S.</td>
</tr>
<tr>
<td>Starch</td>
<td>P&lt;0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>DON × Starch</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

aN.S.=not statistically significant (P>0.05).
Figure 5.9. Hepatic expression of CYP1A in rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days. Values are mean relative fold change + SEM compared to fish fed the diet containing 0.1 ppm DON and 12% digestible starch (3 replicates/treatment).
Figure 5.10. Hepatic expression of UGT1A in rainbow trout (initial average weight=21.9 g/fish) and Nile tilapia (initial average weight=8.1 g/fish) fed the experimental diets for 70 days. Values are mean relative fold change + SEM compared to fish fed the diet containing 0.1 ppm DON and 12% digestible starch (3 replicates/treatment). Significance of the linear orthogonal polynomial contrast is indicated (** P<0.01).
Figure 5.11. Total hepatic UDPGT activity (nmol/min) of rainbow trout fed the diets containing 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (■) for 70 days. Values are means (12 fish/treatment) ± SEM. Significance of the quadratic orthogonal polynomial contrasts across level of DON are indicated for diets containing 12% digestible starch (# P≤0.05) and 24% digestible starch (* P≤0.05).
Figure 5.12. Total hepatic UDPGT activity (nmol/min) of Nile tilapia fed the diets containing 40% crude protein and 12% digestible starch (■) or 32% crude protein and 24% digestible starch (■) for 70 days. Values are means (12 fish/treatment) + SEM. Significance of the linear orthogonal polynomial contrast across level of DON is indicated for diets containing 24% digestible starch (* P≤0.05).
5.5 – Discussion

Two sets of diets, each with a different level of digestible starch (12 or 24% on an as is basis), were formulated to contain the same increasing, graded levels of DON (0.1, 0.7 and 1.3 ppm) from naturally contaminated corn and to meet all known nutrient requirements of rainbow trout and Nile tilapia according to NRC (2011) recommendations. The concentrations of DON present in the experimental diets used here are within the range of those routinely reported in monitoring of a wide variety of agricultural commodities intended for human and animal consumption (Scott, 1997; Streit et al., 2013; Schatzmayr and Streit, 2013; Nährer and Kovalsky, 2014). Consequently, this study represents a practically meaningful contribution to our understanding of species-specific differences in sensitivity to DON.

Rainbow trout and Nile tilapia exhibited significantly different responses to increasing dietary concentrations of DON. The linear decreases in weight gain, TGC, FE, carcass CP content, RN and NRE of rainbow trout with increasing levels of DON in the diets containing either 12 or 24% digestible starch were similar to our earlier results in fish fed diets naturally contaminated with up to 2.6 ppm DON (Hooft et al., 2011). In contrast, no adverse effects on growth performance, carcass composition or nutrient utilization of Nile tilapia were associated with increasing concentrations of DON. The absence of any detrimental effects of DON on growth performance in Nile tilapia is supported by previous observations in channel catfish and common carp (Manning, 2005; Pietsch et al., 2014a,b). However, our findings are in disagreement with those of Tola et al. (2015) who reported significant linear decreases in weight gain, TGC, feed intake and FE of a closely related species, red tilapia (Oreochromis niloticus × O. mossambicus), fed diets containing 0.07 to 1.15 ppm DON from naturally
contaminated wheat. The reason for the differences in the effect of DON on tilapia in these studies is not clear, but may be related to several factors which can modify the expression of toxicity including nutritional and health status prior to exposure, experimental and environmental conditions and/or the mycotoxin contamination profile of the diets (Rotter et al., 1996; Whitlow and Hagler, 2002). Tola et al. (2015) reported the presence of multiple mycotoxins in the experimental diets used in their study including zearalenone (ZON), aurofusarin, rubrofusarin, culmorin, 15-hydroxyculmorin, alternariol (AOH) and alternariol monomethyl ether (AME); nonetheless, several of these metabolites were also detected in the diets used here. Moreover, there is currently little toxicological evidence to suggest that the concentrations of the aforementioned contaminants in the experimental diets in either study could have adversely impacted growth performance (Sauer et al., 1978; Forsell et al., 1986; Rotter et al., 1992; Dvorska et al., 2001; Döll et al., 2010; Alexander et al., 2011).

The tolerance of Nile tilapia to DON relative to rainbow trout was not related to a higher activity of UDPGT in the former species. Rather, the total hepatic UDPGT activity of Nile tilapia was approximately 10-fold lower than that of rainbow trout in general. The reason for this substantial difference could not be elucidated. Extensive metabolism of DON to glucuronide conjugates has been demonstrated in a number of species (Côté et al., 1986; Prelusky et al., 1986a; Eriksen et al., 2003; Meky et al., 2003; Dänicke et al., 2004b, 2005; Goyarts and Dänicke, 2006). Maul et al. (2012) recently indicated that, in addition to UDPGT activity, the pattern of glucuronide metabolites produced also appears to play an important role in species-specific sensitivity to DON. The conjugation activity of rainbow trout liver microsomes towards the formation of a specific glucuronide metabolite, DON-3-β-d-O-glucuronide (DON-3-GlcA), was
lower compared to that of carp liver microsomes (5.8 vs. 14 pmol/min/mg protein), despite higher glucuronidation activity of a reference substrate in rainbow trout (Maul et al., 2012). This suggests that the identification of specific DON-GlcA isomers may be a more suitable indicator of glucuronidation capacity than hepatic UDPGT activity measured using a generic substrate (e.g. \( p \)-NP). Alternatively, the greater sensitivity of rainbow trout to DON in comparison to Nile tilapia may be related to species differences in toxicokinetics and other routes of DON metabolism. For example, the sensitivity of pigs to DON relative to poultry and ruminants has been attributed to rapid adsorption and inefficient microbial transformation of DON to its less toxic de-epoxide metabolite, DOM-1 (Prelusky et al., 1988; Dänicke et al., 2004a,b; Goyarts and Dänicke, 2006). The biological significance of intestinal de-epoxidation in fish is not yet well understood. Guan et al. (2009) were able to demonstrate complete transformation of DON to DOM-1 following incubation of DON with digesta from brown bullhead catfish for 96 hours, but de-epoxidation did not occur in other species including pink salmon and brown trout. Further work is necessary to determine whether differences in de-epoxidation capacity or in the composition of the intestinal microbiota are related to species-specific sensitivity to DON in fish.

It has been established that UDPGA, the availability of which is an important rate-limiting factor in glucuronidation reactions, is predominately derived from hepatic glycogen and thus is inherently connected to carbohydrate metabolism (Dziewiatkowski and Lewis, 1944; Reinke et al., 1979, 1981; Bánhegyi et al., 1988, 1991; Price and Jollow, 1989). Some studies have attempted to manipulate the rate of glucuronidation through alterations in diet composition, with contradictory results. A beneficial impact on the conjugation of drugs
 metabolized primarily by glucuronidation (i.e. increased clearance rate, higher recovery of glucuronides and decreased elimination half-life) was reported in human subjects following consumption of low protein, high carbohydrate diets (Sonne et al., 1989; Pantuck et al., 1991). However, despite an often positive association between the level of digestible carbohydrate in the diet and the hepatic glycogen content in salmonids and other species, we were unable to demonstrate a beneficial effect of increased digestible starch content on the performance of rainbow trout fed diets containing increasing concentrations of DON (Bergot, 1979; Hilton and Atkinson, 1982). Furthermore, increasing the digestible starch content of the diet was not an effective means of significantly increasing the hepatic UDPGT activity in either species in the current study. This may indicate that the provision of a diet containing a high level of digestible starch, which presumably increased the hepatic glycogen content, did not directly influence the supply of UDPGA. In agreement, Hjelle et al. (1986) reported that the depletion of UDPGA in rats treated with high doses of acetaminophen was not due to decreases in the liver concentrations of glycogen or UDP-glucose, but instead, related to an inhibitory feedback effect of NADH accumulation on the activity of UDP-glucose dehydrogenase, an NAD⁺-dependent enzyme responsible for the conversion of UDP-glucose to UDPGA. It appears, therefore, that the influence of nutritional state on glucuronidation is complex and may be regulated at multiple sites within intermediary carbohydrate metabolism prior to UDPGT.

The feeding regime used in this study (i.e. feeding fish to satiety three times daily) may have also contributed to the lack of a positive effect of starch on UDPGT activity. For example, Reinke et al. (1979, 1981) reported that the rate of glucuronidation of $p$-NP was highest in perfused livers obtained from fasted-refed rats compared to either fed or fasted rats.
Interestingly, fasting has been shown to result in the mobilization of liver glycogen stores in several species of fish (Nagai and Ikeda, 1971; Navarro et al., 1992; Meton et al., 2003; Perez-Jimenez et al., 2007). Continuous feeding may have therefore impaired the mobilization of glycogen and its subsequent entry into the uronic acid cycle for UDPGA synthesis.

The relative hepatic expression of CYP450 1A was used to examine the potential contribution of phase I metabolism to the detoxification of DON. In contrast to Sanden et al. (2012), who reported a three-fold increase in the liver CYP450 1A mRNA level of zebrafish fed a diet containing 2.0 ppm DON compared to fish in the control, 0.1, 0.5 and 1.5 ppm DON groups, we did not find a significant effect of DON on CYP1A expression in rainbow trout or Nile tilapia. Similar conclusions indicating a lack of microsomal CYP450-dependent metabolism of DON were described in rats (Morrissey et al., 1985; Côté et al., 1987). Comparatively, exposure of mice to low doses of DON (0.071 or 0.355 mg/kg b.w.) increased the protein levels of CYP450 2B1/2B2, whereas the level of CYP450 1A1/1A2 remained unchanged in animals administered up to 1.774 mg DON/kg b.w. (Gouze et al., 2006). The specificity of the analytical approaches used (e.g. determination of total microsomal CYP450 content vs. isoenzyme expression vs. catalytic activity) may contribute to the differences in the apparent contribution of CYP450 metabolism to the detoxification of DON. Nonetheless, the expression of CYP1A in our study was significantly higher in Nile tilapia compared to rainbow trout, suggesting that species differences in the oxidative metabolism of DON may exist in fish. This could be relevant to glucuronidation since, in compounds which undergo both phase I and phase II metabolism, the rate of oxidation will limit the availability of the substrate for subsequent conjugation (Eacho et al., 1981; Reinke et al., 1981, 1994).
5.6 – Conclusion

Diets containing low, graded levels of naturally occurring DON (0.1, 0.7 and 1.3 ppm) resulted in highly significant decreases in growth performance, carcass CP content and nutrient utilization efficiency in rainbow trout. Conversely, no adverse effects of DON on these parameters were observed in Nile tilapia. To our knowledge, this is the first study to directly compare the response of these species to feed-borne DON. In contrast to our hypothesis, the sensitivity of rainbow trout to DON compared to tilapia was not related to differences in hepatic UDPGT activity. In fact, UDPGT activity of Nile tilapia was approximately 10-fold lower than that of rainbow trout. Moreover, increasing the digestible starch content of the diet as a means to increase the supply of UDPGA, a well-recognized rate-limiting co-factor in glucuronidation, did not significantly increase UDPGT activity in either species. This suggests that the ability of omnivorous species (e.g. Nile tilapia) to more effectively utilize dietary carbohydrates relative to their carnivorous counterparts (e.g. rainbow trout) is not related to differences in sensitivity to DON as originally proposed. The significantly higher relative expression of hepatic CYP1A in Nile tilapia compared to rainbow trout may offer insight into the basis of the difference in sensitivity of omnivorous and carnivorous fish species to DON, but further efforts are needed to investigate this possibility. The species-specific differences in sensitivity of fish to DON observed here might alternatively be explained by the specific pattern of glucuronide conjugates produced or related to differences in the intestinal metabolism of DON. Nonetheless, it appears that the distinct response of these species to DON may provide a unique and effective model for the ongoing elucidation of species-specific sensitivity to mycotoxins.
CHAPTER 6 – DEVELOPMENT OF A MICROPLATE METHOD FOR THE DETERMINATION OF HEPATIC UDP-GLUCURONOSYLTRANSFERASE ACTIVITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND NILE TILAPIA (*OEROCHROMIS NILOTICUS*)

6.1 – Abstract

Hepatic glucuronidation represents an important phase II biotransformation reaction in both mammals and fish. The kinetic characteristics of uridine 5’-diphosphate (UDP) glucuronosyltransferases (UDPGTs) in rainbow trout liver microsomes were examined using *p*-nitrophenol (*p*-NP) as an aglycone and UDP-glucuronic acid (UDPGA) as a glucuronyl donor according to an existing protocol. The kinetic data obtained with varying concentrations of *p*-NP best fit the Hill equation and UDPGT activity was successfully induced following an i.p. injection of β-naphthoflavone (β-NF). The assay was subsequently adapted to a microplate method for determination of UDPGT activity in microsomal samples obtained from rainbow trout as well as Nile tilapia. In contrast to rainbow trout, UDPGT activity of Nile tilapia was best described by Michaelis-Menten kinetics. Based on the linearity of *p*-NP glucuronide formation, a *p*-NP concentration of 0.60 mM and a UDPGA concentration of 6.89 mM were determined to be suitable for assaying UDPGT activity in samples from rainbow trout and Nile tilapia. The microplate method offers several advantages over the conventional assay; most notably it enables the observation of successive kinetics which ensures that enzyme activity is calculated in the most linear (initial) rate of the reaction. It also provides practical advantages in terms of ease-of-use and efficiency. This may be relevant to researchers investigating exposure of wild or farmed fish to environmental or feed-borne contaminants which are substrates of UDPGTs.
6.2 – Introduction

The uridine 5’-diphosphate glucuronosyltransferases (UDPGTs) are a large family of enzymes which catalyze the conversion of endogenous and exogenous compounds to polar, hydrophilic compounds that can be easily eliminated in the bile or urine. Quantitatively, the conjugation of xenobiotics with UDP-glucuronic acid (UDPGA) is the most important phase II detoxification reaction in both mammals and fish, with the liver being the most active site of glucuronidation (Dutton, 1980; Tephly and Burchell, 1990; Clarke et al., 1992; Schlenk et al., 2008). Considerable attention surrounding hepatic UDPGT activities in different fish species has primarily stemmed from the importance of this group of enzymes within an ecotoxicological context. Notably, glucuronide conjugates of several environmental pollutants such as benzo[a]pyrene, naphthalene, phenanthrene and polychlorinated biphenyls (PCBs) have been detected in the bile, urine and tissues of a variety of aquatic species (James, 1987; Clarke et al., 1991, 1992). Ongoing efforts to optimize the economical sustainability of aquaculture feeds through increased utilization of cost-effective plant protein sources has also enhanced the potential for exposure of farmed fish to other known substrates of UDPGTs, including those of anthropogenic (e.g. pesticides and insecticides) and natural (e.g. mycotoxins) origin (Loveland et al., 1984; Schlenk et al., 2008; Berntssen et al., 2010; Hooft et al., 2011; Maul et al., 2012; Pietsch et al., 2013). Consequently, techniques to accurately and efficiently measure UDPGT activity in fish may have diverse applications.

A basic spectrophotometric method based on the glucuronidation of p-nitrophenol (p-NP) has been widely employed to investigate piscine UDPGT activities, most notably in rainbow trout (Castrén and Oikari, 1983, 1987; Koivusaari, 1983; Andersson et al., 1985; Clarke et al.,
1991; Celander et al., 1993; Gadagbui et al., 1996; Taysse et al., 1998; Sturm et al., 1999). However, variations in optimal assay conditions and the kinetics of glucuronidation in this species have been described (Castrén and Oikari, 1983; Sturm et al., 1999). Moreover, despite its relative simplicity, the conventional assay is time- and labour-intensive. The main objectives of this study were therefore to conduct a re-characterization of the enzyme kinetics of UDPGT in rainbow trout using the aforementioned method and to further develop the assay into a more rapid and high-throughput microplate technique. We also sought to extend the application of the microplate method to a second species, Nile tilapia.

6.3 – Materials and methods

6.3.1 – Chemicals

Sucrose, potassium phosphate monobasic (KH$_2$PO$_4$), potassium hydroxide (KOH), 4-nitrophenol (p-nitrophenol; p-NP), uridine 5’-diphosphoglucuronic acid ammonium salt (UDPGA), ethylenediaminetetraacetic acid dipotassium salt dihydrate (K$_2$EDTA), sodium hydroxide (NaOH) and β-naphthoflavone (β-NF) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Trichloroacetic acid (TCA) was obtained from Fisher Scientific (Ottawa, ON, Canada). All reagents were of HPLC, spectrophotometric or reagent grade.

6.3.2 – Experimental animals, treatment and sampling

Rainbow trout (*Oncorhynchus mykiss*) with an average weight of 68.4 ± 14.6 g/fish and Nile tilapia (*Oreochromis niloticus*) with an average weight of 87.5 ± 20.3 g/fish were obtained from the Alma Aquaculture Research Station (Elora, ON, Canada) and Sand Plains Aquaculture (Mossley, ON, Canada), respectively. Groups of 20 fish of each species were randomly
distributed into three 60 L fibreglass tanks, continuously and individually aerated and supplied with water at a rate of 3 L/min. The tanks were maintained within a partial recirculation system (approximately 30% make up water) equipped with gravel biofilters. Water temperature was held constant at 15.3 ± 0.4 °C for rainbow trout and 30.1 ± 0.8 °C for Nile tilapia by injection of hot water into the incoming water line using a solenoid valve controlled by a thermoregulator. Photoperiod was maintained at 12 hours light: 12 hours dark in a windowless laboratory. Fish were acclimated to the experimental conditions for two weeks prior to treatment during which time both species were fed a maintenance ration of a high quality commercial trout feed (Martin Mills Inc., Elmira, Ontario, Canada). All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984) and the University of Guelph Animal Care Committee.

Fish were anesthetized using tricaine methane sulfonate (100 mg/L water) and individually weighed. Two tanks of each species were administered a single intraperitoneal (i.p.) injection of β-NF dissolved in peanut oil (50 mg β-NF/kg body weight) as described by Celander et al. (1993), Gadagbui et al. (1996) and Zhang et al. (1990). Concurrently, one tank of each species received peanut oil only (controls). β-NF, a known polycyclic aromatic hydrocarbon (PAH)-type inducer of UDPGTs, was used in order to ensure that the assay could detect a wide range of enzyme activity. Prior to treatment, fish were fasted for 24 hours. Immediately following injection, fish were transferred to tanks within the same experimental system and monitored. Fish were fed a maintenance ration of the commercial trout feed daily. Ten days post-injection, fish were humanely euthanized by an overdose of tricaine methane sulfonate (200 mg/L water). The livers were carefully excised to prevent rupture of the gall
bladder, washed in phosphate-buffered saline (PBS), blotted, individually placed in 4 mL cryogenic tubes, immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Fish were fasted for 24 hours before sampling.

6.3.3 - Microsome preparation and protein assay

Microsomes were prepared according to Castrén and Oikari (1983) with minor modifications. Pieces were sectioned from thawed livers (1 g where possible), weighed and individually homogenized on ice in 4 vol of 0.25 M sucrose using a Polytron homogenizer (Kinematica AG, Switzerland). Following centrifugation of the homogenate at 4 °C and 12,000 g for 20 min, the supernatant was collected and further ultracentrifuged at 4 °C and 100,000 g for 60 min (Sorvall™ WX Ultra Series, Thermo Fisher Scientific Inc., MA, USA). The resulting supernatant was discarded. The pellet was collected, resuspended in 1 mL of 0.25 M sucrose and manually homogenized on ice using a Potter-Elvehjem glass tissue grinder with a Teflon pestle (Sigma-Aldrich, Oakville, ON, Canada). The microsomal preparations were transferred to 1.5 mL microcentrifuge tubes and stored at −80 °C until analysis. A small aliquot of each sample was stored separately and later used for determination of protein concentration using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer’s specifications.

6.3.4 – Initial conditions and protocol for UDPGT assay A

Hepatic UDPGT activity assays on microsomal preparations from rainbow trout were initially performed as previously described by Castrén and Oikari (1983). This assay will
subsequently be referred to as assay A. Stock solutions of 0.25 M sucrose (used for tissue homogenization), 0.5 M potassium phosphate buffer (pH 7), 0.5 M K$_2$EDTA (in potassium phosphate buffer), 70 mM p-NP (in potassium phosphate buffer), 3% (w/v) TCA and 5 N NaOH were prepared in advance and stored at 4 °C. Immediately prior to conducting the assay, the required volume of a dilution buffer consisting of 6.89 mM UDPGA and 10 mM K$_2$EDTA was prepared using the stock solutions of 0.5 M potassium phosphate buffer and 0.5 M K$_2$EDTA. This dilution buffer was then used to prepare a solution containing 0.35 mM p-NP. Microsomes were thawed, kept on ice and diluted to the desired protein concentration using 0.25 M sucrose. Microcentrifuge tubes containing 200 µL of the diluted microsome preparation and 100 µL of the potassium phosphate buffer containing 0.35 mM p-NP, 6.89 mM UDPGA and 10 mM K$_2$EDTA were vortexed and incubated at 25 °C for 20 min in a water bath. The reaction was stopped by the addition of 0.9 mL of cold TCA (3%, w/v). The tubes were microcentrifuged at 3000 rpm for 10 min and 1 mL of the resulting supernatant was added to 100 µL of 5 N NaOH before determining the absorbance at 400 nm in a spectrophotometer (Ultrospec Plus, Biochrom, Cambridge, UK). A blank consisting of the microsome sample and dilution buffer (6.89 mM UDPGA and 10 mM K$_2$EDTA in 0.5 M potassium phosphate buffer) was used to calibrate the spectrophotometer. An additional blank without UDPGA (p-NP blank) consisting of the microsome sample and 0.5 M potassium phosphate buffer containing 0.35 mM p-NP and 10 mM K$_2$EDTA was used to determine the amount of p-NP glucuronide formation by difference compared to the sample. The concentration of p-NP was determined using an extinction coefficient of 18.6 mM$^{-1}$ cm$^{-1}$ for p-NP generated with a standard curve (r$^2$=0.9999).
6.3.5 – UDPGT assay A optimization

Parameters of interest with regard to the optimization of assay conditions included protein concentration, incubation time, p-NP concentration and UDPGA concentration. When one parameter was varied, the other factors remained constant. Microsomal protein concentration was increased from 0.5 mg/mL to 4.5 mg/mL in increments of 0.5 mg/mL using an incubation time of 20 min and p-NP and UDPGA concentrations of 0.35 mM and 6.89 mM, respectively. Different protein concentrations were achieved by dilution of the same stock microsome preparation using 0.25 M sucrose. Similarly, different incubation times (5, 10, 15, 20, 25 and 30 min) were investigated using constant microsomal protein, p-NP and UDPGA concentrations of 1 mg/mL, 0.35 mM and 6.89 mM, respectively. The concentration of p-NP was varied from 0.025 to 1.2 mM, while the concentrations of UDPGA tested ranged from 0.5625 mM to 18 mM, depending on the experiment. For kinetic experiments involving the optimization of p-NP concentration, the concentration of UDPGA was maintained at 6.89 mM. Likewise, for those experiments in which the concentration of UDPGA was varied, the concentration of p-NP was held constant at either 0.35 mM or 0.60 mM. Different concentrations of p-NP were attained by serial dilution of the highest concentration of p-NP with the dilution buffer (10 mM K$_2$EDTA and 6.89 mM UDPGA in 0.5 M potassium phosphate buffer) or by combining two concentrations of p-NP. For each concentration of p-NP, a blank was included in which the microsome sample had been boiled for 10 min prior to conducting the assay. A similar approach using a dilution buffer consisting of 10 mM K$_2$EDTA and a set concentration of p-NP (either 0.35 or 0.60 mM) in 0.5 M potassium phosphate buffer was used to evaluate the effect of different concentrations of UDPGA on the specific activity of UDPGT.
Optimization of assay A was performed using microsomal preparations from control and β-NF treated rainbow trout. Samples were assayed in duplicate or triplicate.

**6.3.6 – UDPGT microplate assay protocol and optimization**

Hepatic microsomes from control and β-NF treated rainbow trout and Nile tilapia and stock and working solutions were prepared as described above. The reaction was initiated by the addition of 100 µL of 0.5 M potassium phosphate buffer containing the desired concentration of p-NP (0.35 or 0.60 mM), 6.89 mM UDPGA and 10 mM K₂EDTA to 100 µL of microsome sample in a 96-well microtiter plate. Temperature was maintained at 25 °C and absorbance was read at 400 nm every 5 min over a 95-min period using a BioTek PowerWave XS microplate spectrophotometer equipped with KC4 software (BioTek, Winooski, VT, USA). For each sample, blanks consisting of 100 µL diluted microsome and 100 µL dilution buffer were included on the same plate. Samples and blanks were assayed in triplicate. Each sample reading was adjusted by subtraction of the average absorbance of the respective blank. The amount of p-NP remaining at each time point was determined using a standard curve generated by serial dilution of p-NP ($r^2=0.9999$). Subsequently, the amount of p-NP glucuronide present at each interval was determined by difference relative to the amount of p-NP present at the initial reading ($p$-NP$_{initial}$ – $p$-NP$_x$, where $p$-NP$_{initial}$ is the amount of $p$-NP in nmol at the initial reading and $p$-NP$_x$ is the amount of $p$-NP in nmol at a later time, x). Parameters for optimization of the microplate method included microsomal protein concentration and $p$-NP concentration for both species.
6.3.7 – Kinetic evaluation and data analysis

The kinetic parameters $V_{\text{max}}$, $K_m$ (or $K_{0.5}$) and $n$ (where appropriate) were determined by least squares non-linear regression using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Data were fit to the conventional Michaelis-Menten equation (equation 1) and to the Hill equation (equation 2) when substrate activation was suspected, where $v$ represents the reaction rate, $V_{\text{max}}$ is the maximum reaction rate, $[S]$ is the substrate concentration, $K_m$ ($K_{0.5}$) is the substrate concentration at half maximum velocity and $n$ is the Hill coefficient describing cooperativity (where $n>1$ indicates positive cooperativity and $n<1$ indicates negative cooperativity). The quality of fit to a model was assessed by the evaluation of criteria including: (1) visual inspection of the Eadie-Hofstee plots of the data; (2) visual inspection of the residual plots and the sum of squares of the residuals; and (3) the 95% confidence intervals and standard errors of the parameter estimates (Fisher et al., 2000; Motulsky and Christopoulos, 2003; Soars et al., 2003). If an atypical kinetic profile (sigmoidal autoactivation) was suspected following evaluation using these criteria, selection of the Hill model in favour of the Michaelis-Menten model was based on the extra sum-of-squares F test (Motulsky and Christopoulos, 2003; Walsky et al., 2012).

\begin{align*}
\text{Equation (1)} & \quad v = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \\
\text{Equation (2)} & \quad v = \frac{V_{\text{max}} \times [S]^n}{K_{0.5} + [S]^n}
\end{align*}
6.4 – Results

6.4.1 – Optimization of UDPGT assay A

Initial attempts to optimize the microsomal protein concentration and incubation time for the determination of hepatic UDPGT activity of rainbow trout were conducted using 0.35 mM p-NP and 6.89 mM UDPGA according to Castrén and Oikari (1983). Under these conditions, the glucuronidation of p-NP proceeded linearly at protein concentrations in the range of 0.5 to 1.5 mg/mL for a sample obtained from a β-NF treated rainbow trout when incubation time was 20 min (Figure 6.1). Similarly, the reaction rate was linear between 5 and 20 min for samples from both control and β-NF treated rainbow trout when the protein concentration was held constant at 1 mg/mL (Figure 6.2). Consequently, 1 mg/mL and 20 min were selected as the respective optimal microsomal protein concentration and incubation time for assay A.

Visual inspection of the kinetic data obtained with varying concentrations of p-NP according to the established criteria indicated that the Hill equation was a potentially better fit compared to the Michaelis-Menten equation. In particular, the Eadie-Hofstee plots depicted a hook shape consistent with autoactivation kinetics (Figure 6.3, insets). This was supported by statistical evaluation using the extra sum-of-squares F-test (P≤0.05) with the exception of one sample from a β-NF treated fish (Table 6.1). In all cases where the kinetic data best fit the Hill model, the Hill coefficient (n) was indicative of positive cooperativity (n > 1) for both control and β-NF treated fish (Table 6.2). The mean $V_{max}$ for the rate of p-NP glucuronide formation in samples from control (sham injected) rainbow trout was $2.88 \pm 0.102$ nmol/min/mg, while the corresponding mean $K_{0.5}$ for p-NP was $0.096 \pm 0.008$ mM (n=3 fish). In comparison, the mean $V_{max}$ for p-NP glucuronidation and $K_{0.5}$ ($K_m$) for p-NP determined by incubation of β-NF treated
rainbow trout liver microsomes at different concentrations of \( p\)-NP were 8.87 ± 0.456 nmol/min/mg and 0.203 ± 0.029 mM, respectively (n=3 fish). These values represent an approximate 3-fold increase in \( V_{\text{max}} \) and 2-fold increase in \( K_{0.5} (K_m) \) on average of \( \beta\)-NF treated rainbow trout relative to control rainbow trout. Examples of the glucuronidation kinetics attained by varying the concentration of \( p\)-NP using assay A are presented in Figure 6.3. A \( p\)-NP concentration of 0.60 mM was selected for further optimization of the assay since this concentration appeared to be sufficient to attain \( V_{\text{max}} \) in samples from both control and \( \beta\)-NF treated fish.

The glucuronidation of \( p\)-NP in response to manipulation of the concentration of UDPGA displayed Michaelis-Menten kinetics (Table 6.2; Figure 6.4). Visual inspection of the resulting kinetic data, notably the standard errors and 95% confidence intervals of the kinetic parameter estimates, did not justify use of a more complex model. The concentration of UDPGA was increased from a minimum of 0.56 mM to a maximum of 18 mM, depending on the assay. The glucuronidation \( V_{\text{max}} \) values for microsome samples from control rainbow trout were 3.05 ± 0.074 and 4.18 ± 0.083 nmol/min/mg, while the \( K_m \) values of UDPGA for these samples were 1.72 ± 0.179 and 1.62 ± 0.132 mM, respectively. Individual microsome samples analyzed from rainbow trout in which hepatic UDPGT activity had been induced by \( \beta\)-NF injection had \( V_{\text{max}} \) values for \( p\)-NP glucuronidation of 5.78 ± 0.123 and 13.1 ± 0.303 nmol/min/mg with associated \( K_m \) values of 2.09 ± 0.166 and 3.33 ± 0.241 mM UDPGA, respectively (Table 6.2). Based on the \( K_m \) values of the control and \( \beta\)-NF treated samples, the UDPGA concentration of 6.89 mM previously specified by Castrén and Oikari (1983) was determined to be appropriate for assaying samples with a wide range of UDPGT activities.
Figure 6.1. The effect of microsomal protein concentration on the amount of \( p \)-NP glucuronide formed by a \( \beta \)-NF treated rainbow trout liver microsome sample when incubation time was 20 min and the concentrations of \( p \)-NP and UDPGA were 0.35 mM and 6.89 mM, respectively. Values are means ± SEM (2 replicates/microsomal protein concentration).
Figure 6.2. The effect of incubation time on p-NP glucuronide formation by control (A) and β-NF treated (B) rainbow trout liver microsome samples when the concentrations of microsomal protein, p-NP and UDPGA were held constant at 1 mg/mL, 0.35 mM and 6.89 mM, respectively. Values are means ± SEM (2 replicates/incubation time).
Table 6.1. Kinetic parameters for UDPGT activity of rainbow trout liver microsomes with regard to p-NP concentration when UDPGA concentration is held constant at 6.89 mM.

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Assay&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rep&lt;sup&gt;c&lt;/sup&gt;</th>
<th>p-NP concentrations (mM)</th>
<th>$V_{\text{max}}$ (nmol/min/mg)</th>
<th>$K_m$ or $K_{0.5}$ (mM)</th>
<th>$n$</th>
<th>Extra SS F-test&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>A</td>
<td>2</td>
<td>0.1, 0.2, 0.3, 0.45, 0.60, 0.9</td>
<td>4.70 ± 0.174</td>
<td>0.101 ± 0.008</td>
<td>1.88 ± 0.385</td>
<td>0.0308</td>
</tr>
<tr>
<td>C-2</td>
<td>A</td>
<td>2</td>
<td>0.1, 0.2, 0.3, 0.45, 0.6, 0.9, 1.2</td>
<td>2.03 ± 0.098</td>
<td>0.113 ± 0.014</td>
<td>2.79 ± 0.994</td>
<td>0.0477</td>
</tr>
<tr>
<td>C-3</td>
<td>A</td>
<td>3</td>
<td>0.075, 0.15, 0.3, 0.45, 0.6, 0.9</td>
<td>1.91 ± 0.033</td>
<td>0.073 ± 0.003</td>
<td>2.21 ± 0.324</td>
<td>0.0002</td>
</tr>
<tr>
<td>C-4</td>
<td>M</td>
<td>3</td>
<td>0.075, 0.15, 0.3, 0.45, 0.6, 0.9, 1.2</td>
<td>2.94 ± 0.043</td>
<td>0.069 ± 0.003</td>
<td>2.57 ± 0.414</td>
<td>0.0001</td>
</tr>
<tr>
<td>T-1</td>
<td>A</td>
<td>2</td>
<td>0.1, 0.2, 0.3, 0.6, 1.2</td>
<td>8.31 ± 0.723</td>
<td>0.282 ± 0.065</td>
<td>–</td>
<td>0.8779</td>
</tr>
<tr>
<td>T-2</td>
<td>A</td>
<td>3</td>
<td>0.075, 0.15, 0.3, 0.45, 0.6, 0.75, 0.9, 1.2</td>
<td>8.20 ± 0.147</td>
<td>0.164 ± 0.007</td>
<td>1.83 ± 0.123</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T-3</td>
<td>A</td>
<td>2</td>
<td>0.025, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6</td>
<td>10.1 ± 0.499</td>
<td>0.162 ± 0.014</td>
<td>1.53 ± 0.155</td>
<td>0.0019</td>
</tr>
<tr>
<td>T-4</td>
<td>M</td>
<td>3</td>
<td>0.075, 0.15, 0.3, 0.6, 0.9, 1.2</td>
<td>15.8 ± 0.666</td>
<td>0.146 ± 0.014</td>
<td>2.17 ± 0.432</td>
<td>0.0031</td>
</tr>
</tbody>
</table>

<sup>a</sup>C=control (sham injected); T=treated (β-NF). Each sample represents the microsomal fraction obtained from the liver of one fish.
<sup>b</sup>Assay type. A=UDPGT assay A; M=UDPGT microplate assay.
<sup>c</sup>Number of replicates at each substrate concentration.
<sup>d</sup>P-value of the extra sum-of-squares F-test. If $P\leq0.05$, preferred model is Hill; if $P>0.05$, preferred model is Michaelis-Menten.

Table 6.2. Kinetic parameters for UDPGT activity of rainbow trout liver microsomes determined using the Michaelis-Menten equation with regard to UDPGA concentration.

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rep&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p-NP (mM)</th>
<th>UDPGA concentrations (mM)</th>
<th>$V_{\text{max}}$ (nmol/min/mg)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-5</td>
<td>3</td>
<td>0.60</td>
<td>1.125, 2.25, 4.5, 6.75, 9, 13.5, 18</td>
<td>3.05 ± 0.074</td>
<td>1.72 ± 0.179</td>
</tr>
<tr>
<td>C-6</td>
<td>3</td>
<td>0.60</td>
<td>0.5625, 1.125, 2.25, 4.5, 6.75, 9, 13.5, 18</td>
<td>4.18 ± 0.083</td>
<td>1.62 ± 0.132</td>
</tr>
<tr>
<td>T-6</td>
<td>3</td>
<td>0.60</td>
<td>0.5625, 1.125, 2.25, 4.5, 6.75, 9, 13.5, 18</td>
<td>5.78 ± 0.123</td>
<td>2.09 ± 0.166</td>
</tr>
<tr>
<td>T-7</td>
<td>3</td>
<td>0.60</td>
<td>1.125, 2.25, 4.5, 6.75, 9, 13.5, 18</td>
<td>13.3 ± 0.296</td>
<td>3.58 ± 0.246</td>
</tr>
</tbody>
</table>

<sup>a</sup>C=control (sham injected); T=treated (β-NF). Each sample represents the microsomal fraction obtained from the liver of one fish.
<sup>b</sup>Number of replicates at each substrate concentration.
Figure 6.3. Examples of UDPGT enzyme kinetics for control (A, sample C-3) and β-NF treated (B, sample T-2) rainbow trout liver microsomes with regard to p-NP concentration. The corresponding Eadie-Hofstee plots displaying a hook shape representative of atypical kinetics are depicted as insets. Values are means ± SEM (3 replicates/substrate concentration).
Figure 6.4. Examples of UDPGT enzyme kinetics for control (A, sample C-5) and β-NF treated (B, sample T-7) rainbow trout liver microsomes with regard to UDPGA concentration. The corresponding linear Eadie-Hofstee plots characteristic of Michaelis-Menten kinetics are depicted as insets. Values are means ± SEM (3 replicates/substrate concentration).
6.4.2 – UDPGT microplate assay optimization

Glucuronidation of p-NP over time by rainbow trout and Nile tilapia liver microsomes was investigated using 0.35 mM or 0.60 mM p-NP and 6.89 mM UDPGA. The formation of p-NP glucuronide by a sample obtained from a control rainbow trout appeared to be linear over the duration of the experiment (95 min) at a microsomal protein concentration of 1 mg/mL regardless of the p-NP concentration. Product formation by this sample remained linear for the entirety of the assay when the protein concentration was increased to 2 mg/mL and the p-NP concentration was 0.60 mM. Expectedly, the time during which the rate of p-NP glucuronidation was linear decreased in β-NF treated rainbow trout microsomes compared to the control sample. Glucuronide formation remained linear for a longer duration in β-NF treated rainbow trout microsomes at 0.60 mM p-NP compared to 0.35 mM p-NP and when the microsomal protein concentration was 1 mg/mL as opposed to 2 mg/mL. The rate of p-NP glucuronide production reached a plateau after approximately 40 min in the microsome sample obtained from β-NF treated rainbow trout when the protein concentration was 2 mg/mL (Figure 6.5). In agreement with the findings from assay A, kinetic parameters for a control and β-NF treated sample determined using the microplate assay were best estimated using the Hill equation (Table 6.1).

The formation of p-NP glucuronide by microsomes obtained from β-NF treated Nile tilapia liver samples appeared to be fairly linear over 95 min for all conditions tested (0.35 or 0.6 mM p-NP and 1, 2 or 3 mg/mL microsomal protein); however, some samples assayed from fish used in a growth trial (data not shown) demonstrated a latent UDPGT activity between 0 and 20 min. Given this observation, a higher microsomal protein concentration of 2 mg/mL was
used for Nile tilapia compared to rainbow trout and specific activity was determined using incubation times greater than 20 min according to the linearity of p-NP glucuronide formation for individual samples (usually between 40-60 min). Kinetic data for liver microsomes sampled from Nile tilapia were generated by varying the concentration of p-NP and fit to the Michaelis-Menten equation (Figure 6.7). $V_{\text{max}}$ values corresponding to the control, β-NF treated and untreated (i.e. fish from a growth trial) samples were $1.46 \pm 0.333$, $3.17 \pm 0.950$, $1.59 \pm 0.174$ nmol/min/mg, respectively. The related $K_m$ values for the control, treated and untreated samples were $0.676 \pm 0.236$, $1.32 \pm 0.517$ and $0.453 \pm 0.105$ mM p-NP, respectively (Figure 6.7). In both the control and treated samples, a p-NP concentration of 0.9 mM appeared to inhibit the specific activity of UDPGT. Therefore, 0.60 mM p-NP was selected as a suitable concentration for determination of hepatic UDPGT activity in Nile tilapia using the microplate method.
Figure 6.5. Formation of p-NP glucuronide over time by control and β-NF treated rainbow trout liver microsomes incubated with 0.35 (A) or 0.60 (B) mM p-NP and 6.89 mM UDPGA using a microplate assay. (O) control, 1 mg microsomal protein/mL; (△) control, 2 mg microsomal protein/mL; (square) treated, 1 mg microsomal protein/mL; (×) treated, 2 mg microsomal protein/mL. Values are means ± SEM (3 replicates/sample).
Figure 6.6. Formation of p-NP glucuronide over time by β-NF treated Nile tilapia liver microsomes incubated with 0.35 (A) or 0.60 (B) mM p-NP and 6.89 mM UDPGA determined using a microplate assay. (○) 1 mg microsomal protein/mL; (□) 2 mg microsomal protein/mL; (△) 3 mg microsomal protein/mL. Values are means ± SEM (3 replicates/sample).
Figure 6.7. Michaelis-Menten enzyme kinetics for control (●), β-NF treated (■) and untreated (▲) Nile tilapia liver microsome samples determined using different concentrations of p-NP with a microplate assay method. Values are means ± SEM (3 replicates/substrate concentration).
6.5 – Discussion and conclusion

This study presents an updated evaluation of UDPGT activity in rainbow trout using an established protocol in an attempt to verify optimal assay conditions and kinetic parameters. The conventional assay was subsequently developed into a more efficient microplate method which was shown to be effective in detecting UDPGT activity in hepatic microsomal preparations from rainbow trout as well as Nile tilapia.

We observed that when the concentration of p-NP was varied, UDPGT activity was usually best fit to the Hill equation (Hutzler and Tracy, 2002). The Hill equation, describing autoactivation kinetics, implies allosteric regulation in which substrate-enzyme interactions may occur at more than one site per enzyme. When the Hill coefficient is greater than one (n>1), as reported here, positive cooperativity is suggested. That is, the binding of the first substrate molecule can enhance the affinity of the enzyme for additional substrate (Kolodziej et al., 1996; Houston and Kenworthy, 2000; Hutzler and Tracy, 2002). This type of kinetics for glucuronidation of endogenous substances and drugs including bilirubin, estradiol and acetaminophen has previously been demonstrated in isolated rat hepatocytes and human liver microsomes (Bruni and Chang, 1999; Fisher et al., 2000; Walsky et al., 2012). Sacco et al. (2008) also found that the aglycone kinetics for the hepatic and intestinal glucuronidation of certain PCBs in channel catfish could be best characterized using the Hill equation; nonetheless, non-Michaelis-Menten kinetics have not been widely considered in studies of UDPGT activity, particularly in fish. Interestingly, when the microsomal fraction from Nile tilapia was incubated with different concentrations of p-NP using the adapted microplate assay, the Michaelis-Menten equation provided the best fit of the kinetic data. This may suggest variability among
species in terms of UDPGT structure and function. However, Houston and Kentworthy (2000) note that metabolic kinetics may be substantially affected by several factors including the enzyme source (e.g. purified/recombinant enzymes vs. microsomal preparations) and a paucity of data points. In agreement, we suggest that continued and more in-depth studies are necessary to confirm the sigmoidal UDPGT kinetics reported in this experiment.

The $V_{\text{max}}$ and $K_{0.5}$ values with varying $p$-NP concentrations were increased 2- to 3-fold in rainbow trout treated with $\beta$-NF compared to the sham-injected controls. The magnitude of induction of UDPGT activity with $\beta$-NF treatment was comparable to previous reports in rainbow trout (Andersson et al., 1985; Celander et al., 1993). Similarly, i.p. injection with $\beta$-NF resulted in an approximate 2-fold induction of the $V_{\text{max}}$ and $K_m$ values in tilapia which was in fairly good agreement with a 1.8-fold increase in the specific activity of UDPGT observed by Gadagbui et al. (1996). Consequently, we were able to verify that the assay conditions were suitable to determine a wide range of UDPGT activities. The $p$-NP and UDPGA concentrations selected for the standard assay were 0.60 and 6.89 mM, respectively. These conditions were chosen because they appeared to be sufficient to attain $V_{\text{max}}$ and resulted in linear formation of $p$-NP glucuronide over time in both rainbow trout and Nile tilapia samples using the microplate method. A higher microsomal protein content of 2 mg/mL for Nile tilapia compared to 1 mg/mL for rainbow trout is suggested since the formation of $p$-NP glucuronide did not occur immediately following incubation in several microsomal samples obtained from Nile tilapia (data not shown). Latency of UDPGTs (i.e. lower activities of UDPGTs in native microsomes compared to microsomes treated with membrane-disrupting agents) is a well-characterized phenomenon related to the position of the catalytic site of the enzyme in the lumen of the
endoplasmic reticulum (Tephly and Burchell, 1990; Bánhegyi et al., 1993; Fulceri et al., 1994). The use and optimization of membrane-disrupting agents was outside the scope of this study; however, the latent activity of UDPGT in microsome samples from Nile tilapia compared to those from rainbow trout may suggest that incubation conditions specific to each species are required. Nonetheless, the specific UDPGT activities of samples obtained from Nile tilapia using the microplate method were within the range of those previously described for this species (Gadagbui et al., 1996).

The development of a microplate method offers several advantages over the conventional assay. In particular, the microplate assay enables observation of successive kinetics rather than the use of fixed time points. As a result, the most linear (initial) rate of the reaction can be identified for individual samples. In contrast, the conventional assay is based on an initial linearity study which is used to determine a fixed incubation period. Collier et al. (2000) noted that, compared to a predetermined incubation time, the ability to observe the initial rate of the reaction using a microplate method resulted in considerably more accurate estimations of $V_{\text{max}}$ and $K_m$ for UDPGT. The microplate assay also allows for each sample to serve as its own reference blank (i.e. $p$-NP glucuronide can be calculated from an absorbance reading of the same sample at two different time points within the linear range of the reaction). Furthermore, the microplate method is less labour-intensive and more efficient than the conventional assay since multiple samples can be analyzed simultaneously.

In conclusion, this study resulted in the determination of assay conditions appropriate for the determination of UDPGT activity in microsomal preparations from rainbow trout and Nile tilapia. We were also able to successfully adapt an existing spectrophotometric protocol
for the determination of hepatic UDPGT activity in these species to a simpler and more efficient microplate method. This may have practical implications for the determination of UDPGT activity in fish as it relates to the regulation of environmental contaminants and the potential presence of compounds which are substrates of UDPGTs in aquaculture feeds.
CHAPTER 7 – GENERAL DISCUSSION

The potential risk of exposing farmed fish to mycotoxins is an emerging concern in aquaculture nutrition due, primarily, to the increased inclusion of plant-based ingredients in feeds for aquatic species (Manning, 2005; Spring and Fegan, 2005; Sissener et al., 2011; Sanden et al., 2012; Hauptman et al., 2014). DON, a trichothecene mycotoxin produced by fungal species belonging to the genera *Fusarium*, is of particular concern in animal production due to its widespread distribution in a number of geographical regions, high prevalence in cereal grains and resistance to routine processing methods (e.g. extrusion; Grove, 1988, 1993, 2000; Hazel and Patel, 2004; Pestka, 2007). To date, the limited work concerning the effects of DON on fish indicates that considerable species differences in sensitivity exist. However, the basis of species-specific sensitivity to DON in fish has not yet been elucidated. Moreover, fundamental questions surrounding the toxicological relevance of co-occurring mycotoxins in naturally contaminated sources of DON and the use of commercially available products to mitigate the adverse effects of DON on sensitive finfish species (e.g. rainbow trout) remain to be addressed. This thesis provides valuable insight into these unresolved issues.

The results presented in Chapter 3 indicate that the depressive effects of diets predominantly contaminated with DON and other *Fusarium* mycotoxins (e.g. ZON, 15-ADON) on the growth performance, carcass composition and nutrient utilization of rainbow trout are most likely exclusively associated with DON. To the best of our knowledge, this appears to be the only study to date examining the potential of toxicological interactions between naturally co-occurring *Fusarium* mycotoxins in fish. Co-contamination of feed and food ingredients with
multiple mycotoxins is quickly becoming an issue of urgent concern. In 2013, analysis of more than 4,000 commodity samples (from 50 countries) intended for use in animal feed revealed the presence of more than one mycotoxin in nearly half (45%) of the tested samples. Notably, Streit et al. (2012) highlighted the frequent co-occurrence of DON and other *Fusarium* mycotoxins including 3-ADON and 15-ADON, T-2 toxin, HT-2 toxin, fumonisins, moniliformin and ZON. However, relatively little information regarding the biological consequences (e.g. effects on growth, nutrient utilization and health indices) of feed contaminated with multiple *Fusarium* mycotoxins is currently available (reviewed by Grenier and Oswald, 2011). The paucity of data on toxicological interactions between co-occurring mycotoxins has impeded the establishment of regulatory and/or legislative guidelines pertaining to multiple contaminants (Streit et al., 2012; FAO, 2004). The work presented here may therefore be helpful in the development or revision of mycotoxin regulations for fish and other species. Our observations also provide an important precedent for the design and accurate interpretation of future experiments investigating the effects of DON on rainbow trout and other sensitive fish species, particularly those utilizing naturally contaminated ingredients as a practical and cost-effective alternative to purified compounds.

The histopathological effects of the experimental diets were examined in order to better understand the basis of the high sensitivity of rainbow trout to DON (Chapter 3). Despite the absence of distinct pathological lesions, significant increases in the number of dead (apoptotic/necrotic) cells in the pyloric caeca and significant decreases in the number of mitotic cells in the pyloric caeca and liver were generally associated with increasing dietary levels of purified DON (0-2.1 ppm) or DON from naturally contaminated corn (0-5.9 ppm). Interestingly,
these findings are in direct agreement with the ability of DON to induce cell death and interfere with normal cell cycle progression as a result of MAPK activation (Tiemann et al., 2003; Zhou et al., 2005b; Yang et al., 2008; Bensassi et al., 2009; Pestka, 2010b). These results imply that rainbow trout could be a potentially valuable animal model for studies examining the cellular, molecular and metabolic effects of DON. Relatedly, rainbow trout have been widely used to investigate mechanisms of aflatoxicoses and human carcinogenesis (Eaton and Gallagher, 1994; Williams et al., 2003). Several advantages of rainbow trout as a non-mammalian model species in cancer research have been described including a low per diem cost relative to other species (e.g. rodents), high sensitivity to various carcinogens and the ability to conduct studies with large numbers of animals in order to address statistically challenging questions (Williams et al., 2003). The highly repeatable effects of relatively low concentrations of DON (< 2 ppm) on the growth performance of rainbow trout (Hooft et al., 2011; Chapters 3, 4 and 5) and the characterization of cellular changes consistent with those established in mammals (Chapter 3) suggest that this species may likewise be useful as an animal model in mechanistic studies of DON toxicity.

Commercial mycotoxin adsorbents and biotransforming agents are commonly included in animal feeds in order to mitigate the effects of a variety of mycotoxins. However, their use in fish feeds contaminated with Fusarium mycotoxins has been largely without concrete evidence. Therefore, in Chapter 4, we evaluated the efficacy of a commercial feed additive (CFA) in preventing the adverse effects of DON on rainbow trout. Inclusion of the CFA at the recommended rate (2 g/kg feed) did not minimize the adverse effects of diets containing increasing, graded levels of DON (up to 2.0 ppm) on growth performance, body composition or
nutrient utilization of rainbow trout. We hypothesized that the development of the CFA under \textit{in vitro} conditions simulating the gastrointestinal tract of homeothermic species (e.g. temperature, pH, intestinal transit time) may be a major factor associated with its ineffectiveness in rainbow trout (Fuchs et al., 2000, 2002; Avantaggiato et al., 2004; Yiannikouris et al., 2004a,b, 2006). It appears that further testing of currently available anti-mycotoxin feed additives in sensitive fish species such as rainbow trout is required to provide aquaculture feed manufacturers with accurate information. Feed additives marketed for mammalian species cannot necessarily be assumed to effectively counteract mycotoxins in fish. The current study provides an example of a robust experimental design for the continued assessment of additional products to counteract the adverse effects of \textit{Fusarium} mycotoxins in fish (Boudergue et al., 2009). This study also highlights the importance of utilizing a wide variety of pre- and post-harvest mycotoxin mitigation strategies. Moreover, accurate screening of ingredient lots and science-based industry standards regarding the acceptable level of DON contamination of salmonid feeds are critical to avoid mycotoxin-related economic losses in aquaculture production.

In Chapter 5, we aimed to evaluate whether or not sensitivity to DON is related to differences in glucuronidation (assessed as UDPGT activity) associated with the greater capacity of an omnivorous species, Nile tilapia, to effectively utilize dietary carbohydrates relative to a carnivorous species, rainbow trout. In contrast to our hypothesis, UDPGT activity did not explain the species-specific response to DON nor was the effect of DON on either species influenced by the digestible starch content of the diet. Nile tilapia were unaffected by diets containing graded levels of naturally occurring DON (0.1, 0.7 and 1.3 ppm) which otherwise
resulted in significant adverse effects on growth performance, carcass composition and nutrient utilization parameters of rainbow trout. To our knowledge, this is the only study of its kind to directly compare the effects of DON on two fish species of high commercial value. Fish feed manufacturers are faced with the considerable challenge of formulating diets for a large number of species to a wide variety of nutritional specifications. Within this context, the information generated as a result of this study may be useful in establishing industry recommendations for maximum acceptable levels of DON in ingredients and finished feeds for omnivorous and carnivorous species. As demonstrated in Chapter 4, currently available feed additive solutions for mycotoxins may not be effective in sensitive fish species such as rainbow trout. Consequently, consideration of the impact of interactions between contaminants and the nutrient composition of the diet on metabolism is highly relevant to the potential development of efficacious nutritional mitigation strategies. Furthermore, the profound differences in the response of rainbow trout and Nile tilapia to DON may make these species useful animal models in future studies focused on the elucidation of metabolic factors responsible for species-specific sensitivity to DON and other mycotoxins. Such efforts could ultimately lead to the improvement of feed additives for sensitive fish species.

The work presented here is a comprehensive and broad effort aimed at elucidating the underlying physiological and metabolic factors associated with the high sensitivity of rainbow trout to the common *Fusarium* contaminant, DON. Figure 7.1 provides a summary of our results pertaining to the effects of DON on the growth rate of rainbow trout and Nile tilapia. These findings could be used in order to generate risk assessment analyses for the purposes of predicting the economic impacts of DON on aquaculture production and setting regulatory
standards (Wu, 2004, 2007). This would provide feed manufacturers with a tool which could then be integrated into feed formulation programs, enabling evidence-based assessment of the DON risk associated with any particular ingredient lot. In addition to this highly practical application of our work, we have demonstrated that rainbow trout may be a suitable animal model for studying DON. However, ongoing efforts are needed to confirm that the cellular effects of DON (increased and decreased numbers of dead and mitotic cells, respectively) observed in rainbow trout are related to molecular mechanisms established in other species and cell lines (i.e. activation of MAPKs). Similarly, more work is required to evaluate the toxicological interactions between different combinations of *Fusarium* mycotoxins at different doses. This could be accomplished through *in vivo* studies with rainbow trout using diets containing purified mycotoxins similar to those formulated in Chapter 3. Finally, comparative studies using different fish species may be valuable in better understanding the metabolic and toxicokinetic factors responsible for species-specific sensitivity to DON. For example, additional studies comparing rainbow trout and Nile tilapia (or other apparently tolerant species such as channel catfish) could allow for more detailed insight into the relevance of the particular pattern of glucuronide conjugates (e.g. DON-3-GlcA vs. DON-15-GlcA) to species-specific sensitivity to DON, the role of phase I metabolism (CYP450s) in the detoxification of DON and the metabolic interactions between intestinal microbiota and mycotoxins.
Figure 7.1. Summary of the effects of diets containing purified DON and DON from naturally contaminated (NC) corn on the growth rate (expressed as TGC) of rainbow trout and Nile tilapia. The findings are for rainbow trout unless otherwise specified.
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