FEEDING *FUSARIUM*-INFECTED WHEAT TO YELLOW MEALWORM LARVAE (*TENEBRIO MOLITOR*) TO PRODUCE A SAFE, REPLACEMENT PROTEIN SOURCE FOR ANIMAL FEED

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ABSTRACT

Various insect species including the yellow mealworm (*Tenebrio molitor*) could be an alternative and sustainable source of protein for animal feed. There is evidence that yellow mealworm larvae can utilize deoxynivalenol (DON)-contaminated wheat as a food source without sequestering it, producing a safe protein ingredient. This study aimed to determine the potential accumulation of DON in *T. molitor* larvae reared on *Fusarium*-infected wheat containing high levels of DON and investigate the effects of DON exposure on production, survival and nutritional traits on the larvae. Wheat containing 200 µg/kg DON was used as control diet. A different source of wheat was sorted into six fractions and mixed to obtain three levels of DON for low (2,000 µg/kg), medium (10,000 µg/kg) and high (12,000 µg/kg) treatments. Each treatment was replicated five times with 300 or 200 mealworms per replicate for the feeding and breeding trials, respectively. Trial termination was determined when the first two pupae were observed (32-34 days). There was no difference in the levels of DON detected in the larvae between treatments and ranged from 121.8±19.3 to 136.4±40.5 µg/kg (P=0.883). Excretion of DON in pooled frass samples was 131.0, 324.0, 230.4 and 742.1 µg/kg for control, low, medium and high, respectively. The concentrations of 3-acetyldeoxynivalenol (3-ADON) detected in frass ranged from 279.5 to 326.4 µg/kg, whereas levels in larvae ranged from 65.3 to 66.2 µg/kg and were from undetectable to 204.9 µg/kg in wheat. Nutritional analysis on pooled samples from both trials showed maximum levels of crude protein (CP) of 52% and crude fat (CF) of 36%. Ash, fiber, chitin, fatty-acids and amino-acids content were consistent across treatments. Survival was greater than 96% for all life stages in both trials. In the feeding trial, average daily gain (ADG) ranged from 1.9±0.1 to 2.1±0.1 mg/day per mealworm. Less than 1.2% of the ingested DON was accumulated by larvae when they consumed *Fusarium*-infected wheat containing levels up to 12,000 µg/kg. These results along with the lack of effect on the nutritional profile, survival, or production traits, supports using DON-contaminated wheat in large-scale production of mealworms to produce a sustainable, safe protein source.

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LIST OF ABBREVIATIONS

15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
AB	Alberta
ADF	acid detergent fiber
ADG	average daily gain
ADIP	acid detergent insoluble nitrogen
AF	aflatoxin
AFB_1	aflatoxin B1
AOAC	Association of Official Analytical Chemists
BEA	beauvericin
CF	crude fat
CFIA	Canadian Food Inspection Agency
CGC	Canadian Grain Commission
CP	crude protein
DM	dry matter
DOM-1	diepoxy-deoxynivalenol
DON	deoxynivalenol
DON-15G	deoxynivalenol-15-glucoside
DON-3G	deoxynivalenol-3-glucoside
ECI	efficiency of conversion of ingested food
ENN	enniatin
FAO	Food and Agriculture Organization
FCR	feed conversion ratio
FDK	*Fusarium* damage kernels
FDL	freeze dried larvae
FHB	*Fusarium* head blight
FI	feed intake
FUM	fumonisin
HT-2	HT-2 toxin

IARC	International Agency for Research on Cancer
MB	Manitoba
MON	moniliformin
NDF	neutral detergent fiber
NIT	near-infrared transmittance
NIV	nivalenol
ODL	oven-dried larvae
OTA	ochratoxin A
PER	protein efficiency ratio
RH	relative humidity
SK	Saskatchewan
T-2	T-2 toxin
Z3-12:Ac	Z-3-dodecenyl acetate
ZEA	Zearalenone

CHAPTER 1

1. GENERAL INTRODUCTION

Novel, sustainable and inexpensive sources of animal protein are essential to supply the world's growing population. As the demand for human food increases, livestock producers need to produce more animal protein to satisfy the increased demand. Current sources of protein for animal feed such as fish-meal and soybean-meal are expensive and require the overuse of natural resources or a large land base, respectively (Zhao *et al.*, 2016). For these reasons, more sustainable sources of protein for animal feed need to be generated, and thus aid the production of livestock at a lower cost.

On the Canadian priaries, *Fusarium* fungi in cereal grains is an ongoing issue for crops and livestock farms and negatively impacts the economy. When *Fusarium* spp. infect cereal crops they cause *Fusarium* Head Blight (FHB), a disease that leads to losses in grain yield and quality due to the presence of *Fusarium*-damaged kernels (FDK; Dill-Macky and Jones, 2000). Besides the economic losses associated with down-graded and salvage wheat, *Fusarium* is also associated with the production of mycotoxins, secondary metabolites that can cause acute and chronic disease (mycotoxicosis) if consumed by livestock. Often, FDK are blended with high quality grain to decrease the risk of mycotoxicosis and allow the crop to be sold. In years where *Fusarium* infection is high, tons of salvage wheat are produced and are typically burnt or buried, which also adversely impacts the environment.

Historically, insects have formed a natural part of animal diets, especially in free range poultry and fish. The ability of some insects to grow and reproduce on foods that are unusable to vertebrates makes them excellent candidates to replace the conventional protein sources used for animal feed. Insects contain high nutritional value when reared on organic by-products such as grain industry residues and ethanol plant wastes (Ramos-Elorduy *et al.*, 2002a). Hence, insects could possibly be a cheaper protein replacement if reared on unpriced wheat infected with *Fusarium* spp. or contaminated with the mycotoxin, deoxynivalenol (DON). Research has shown that insects such as yellow mealworm larvae (*Tenebrio molitor*) raised on wheat contaminated with high levels of DON were comprised of ~50% crude protein and ~37% crude fat (Guo *et al.*, 2014; Van Broekhoven *et al.*, 2017). Moreover, these yellow mealworm larvae revealed undetectable levels of DON (Guo *et al.*, 2014; Van Broekhoven *et al.*, 2017). These findings suggest that the mealworm larvae could potentially be fed *Fusarium*-infected/mycotoxin-contaminated wheat and, if produced on a large scale, generate an alternative source of protein for animal feed. Hence, this thesis research aims to evaluate the potential use of *Fusarium*-

infected/DON-contaminated wheat in rearing yellow mealworm larvae to produce a safe and sustainable protein source for animal feed.

CHAPTER 2

2. LITERATURE REVIEW

2.1 The global impact of mycotoxins.

Cereals represent a major portion of the food available to feed the world's human population. World cereal production has increased substantially to meet the demand for animal and human food. In 2017, 2.65 billion tonnes of cereals such as wheat, barley and sorghum were produced worldwide (FAO, 2018). Despite the steady increase in cereal production over the last decade, extensive losses have occurred as a result of fungal diseases, devaluing the wheat or even assigning it to salvage which has no commercial value. Fungal infestation in cereal crops became not only harder to control but the most expensive problem for the grain industry due to the production of mycotoxins (IARC, 2012). Multiple environmental factors are associated with the presentation of mycotoxins globally. As proposed by Moretti *et al.* (2018), the rise in moisture and precipitation along with climate change contributed to the increased incidence and dissemination of fungal pathogens and their associated mycotoxins between the period of 2007 and 2017. In 2010, approximately 13% of the global cereal supply was compromised due to the presence of DON, zearalenone (ZEA), beauvericin (BEA), enniatin (ENN) and moniliformin (MON) produced by *Fusarium* spp. (Tittlemier *et al.*, 2013). Thirty percent of this supply was assigned a lower grade or was designated salvage reducing export potential (Gräfenhan *et al.*, 2013). By 2017, the incidence of these mycotoxins had increased substantially; for instance, DON and ZEA had risen to 58% and 46% respectively (Biomin, 2017). The 2017 Biomin survey calculated that 97% of 18,757 samples analyzed worldwide contained ten or more mycotoxins, with *Fusarium* toxins present in all of them (Biomin, 2017). The majority (75%) of these were positive for DON and its derivatives 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and deoxynivalenol-3-glucoside (DON-3G) at levels higher than 1,000 µg/kg.

By March 2018 the situation had worsened. The Biomin mycotoxin survey revealed that the occurrence of mycotoxins had increased substantially in comparison with the previous year. Ninety percent of 3,857 worldwide samples analyzed detected at least one mycotoxin, with DON and fumonisin (FUM) being the most prevalent followed by ZEA, T-2 toxin (T-2), aflatoxins (AF) and ochratoxin A (OTA; Biomin, 2018). Levels as high as 41,157 for DON and 3,049 µg/kg for ZEA were detected (Biomin, 2018). Additionally, 68% of these samples contained multiple mycotoxins (co-occurring mycotoxins).

With the increasing co-occurrence of multiple mycotoxins globally, efforts aim to investigate control strategies to prevent fungal infection in pre-harvest and stored crops, and to ameliorate the negative impact that mycotoxins represent in grain-based products. Economic losses associated with rejected shipments, animal and human illness, and downgraded/salvage grain have challenged researchers to find alternatives to dispose of the infected or contaminated material that cannot be consumed.

2.2 *Fusarium* mycotoxins

Among the multiple fungal species that produce mycotoxins, *Fusarium* represents one of the most devastating in terms of crops yield costs in Canada and many other countries. Currently, more than 170 species of *Fusarium* have been reported, and it is hypothesised that at least double this number have not yet been discovered (Jimenez Garcia *et al.*, 2018). About ninety species of *Fusarium* have been isolated worldwide from raw and grain-based material, and have the capacity to produce multiple mycotoxins (Dahl and Wilson, 2018). *Fusarium* species are widespread and causative agents of FHB disease in wheat, barley and durum. The negative impact associated with FHB are due to the production of FDK characterized by shriveled kernels that are chalky white-pinkish in colour (Dill-Macky and Jones, 2000). Commonly, crops with high numbers of FDK also contain high levels of *Fusarium* mycotoxins, although kernels infected with the mold do not always contain mycotoxins.

Fusarium mycotoxins are mostly trichothecenes; however, ZEA and FUM are also produced and are not within the trichothecene family of mycotoxins. Trichothecenes are characterized by the tetracyclic 12,13-epoxy-trichothec-9-ene skeleton, and classified into groups A, B, C and D (Plumlee, 2004). Toxicity associated with trichothecenes include the disruption of DNA, RNA and protein synthesis inducing apoptosis in eukaryotic cells (Gupta, 2012). DON and nivalenol (NIV) are type B trichothecenes that commonly contaminate wheat, and are important due to the high risk of toxicity in domestic animals that consume contaminated feed (Jimenez Garcia *et al.*, 2018). However, mycotoxins ZEA (an oestrogenic mycotoxin) and FUM (a group of structurally related compounds) are also commonly associated with trichothecenes type B adding to the consequences of DON and NIV in livestock production and health (Gupta, 2012).

2.2.1 *Fusarium* species and occurrence in Western Canada

The loss in revenue for the producer, the cereal sector and the Canadian economy can be considerable in years where *Fusarium* infection is high. The Western Canadian provinces, Alberta (AB), Saskatchewan (SK), and Manitoba (MB), yield approximately 80% of the grain produced in Canada (Gräfenhan *et al.*, 2013). In 2016, two million metric tonnes of wheat and durum were devaluated to salvage due to it containing greater than 10% FDK, representing a loss of 600 million dollars to Canada's economy (Canadian Grain Comission, 2016). Despite efforts to prevent the spread of *Fusarium* from contaminated crops in the northern US into Canada, it was inevitable and difficult to control as environmental conditions favoured the fungal growth (Gräfenhan *et al.*, 2013).

In AB and southern SK, Tittlemier *et al.* (2013) reported that *F. graminearum* and *F. culmorum* are the major species that produce DON and its derivative forms, 3-ADON and 15-ADON. Additional *Fusarium* spp. such *F. avenaceum, F. pseudograminearum, F. crookwellense, F. acuminatum, F. asthroporoides, F. equiseti* and *F. poae* have also been isolated from FDK in Canada, and mycotoxins ZEA, NIV and Fuseron X also detected (Tittlemier *et al.*, 2013, Turkington *et al.*, 2011, Clear and Patrick, 2010).

2.2.2 DON and derivatives

Deoxynivalenol (12,13-epoxy-3,4,15-trihyroxytrichothec-9-en-9-one), also known as vomitoxin, is the major trichothecene produce by *Fusarium spp.* (*F. graminearum* and *F. culmorum*), and one of the most common mycotoxins in grain crops (Sobrova *et al.*, 2010). The production of DON is strongly associated with the presence of *Fusarium* spp. Thus, environmental conditions that favour the proliferation of *Fusarium*, also benefits the production of DON (Tittlemier *et al.*, 2013). Deoxynivalenol is resistant at temperatures as high as 350 °C, which make it highly stable to heat treatments, and therefore a common contaminant in finish products. The toxicokinetics of DON allow it to undergo absorption, distribution, metabolism and excretion, if consumed by the animal (Gupta, 2012). Importantly, DON is metabolized into conjugated metabolites and excreted in bile and urine two to three days after ingestion (Sobrova *et al.*, 2010; Plumlee, 2004). Many metabolic processes may be involved in the biotransformation of DON into its derivatives forms. In mammals, DON has been biotransformed via glycosylation yielding DON-3G and deoxynivalenol-15-glucoside (DON -

15G), acetylation yielding 3-ADON and 15-ADON and gut microbiota de-epoxidation yielding deepoxy-deoxynivalenol (DOM-1; Maresca, 2013). In plants, the production acetylated, glycosylated and de-epoxidated derivatives have also been described by Ran *et al.* (2013). However, two additional pathways lead to the production of 3-oxo-DON and 3-epi-DON via oxidation and epimerization, respectively (Ran *et al.*, 2013).

In Canada, the Canadian Food Inspection Agency (CFIA) required grain-related industries to test crops for the concentration of mycotoxins. If levels exceed the regulatory limits imposed for mycotoxins in single ingredients and feed, the grains need to be brought down into acceptable levels or it is deemed salvage and having no value. Unfortunately, levels of DON and its acetylated derivatives have increased considerably in the last few years, even though mitigation actions in pre-harvest, harvest and store conditions have been taken by producers and governments (Biomin, 2017; Qian *et al.*, 2009). Remarkably, DON levels in North American wheat have tested from 700 µg/kg in 2000 to 7,900 µg/kg in 2013 and 6,467 µg/kg in 2017 (Biomin, 2017; Clear and Patrick, 2010; Gräfenhan *et al.*, 2013).

2.3 Impact of DON on animal production.

Contaminants of grains such as deoxynivalenol negatively impacts the livestock sector. From contaminated raw material and processed feed to mycotoxicosis in animals and residues in animal-derived products, DON can also negatively impact livestock production.

2.3.1 Occurrence of DON in animal feed.

The occurrence of mycotoxins in feed will vary according to the geographical area where the grain was grown and feed was produced (Placinta *et al.*, 1999). As result, the incidence of DON and other mycotoxins in feed may be unique in different parts of the globe. According to Monbaliu *et al.* (2009), DON, 3-ADON, 15-ADON and FUM are the most common contaminants in sow feed, wheat and maize samples from Hungary, Denmark and Czech Republic. In another study, Zachariasova *et al.* (2014) found that DON combined with its acetylated/glycosylated forms were frequently found in feedstuff samples (maize, alfalfa silage and feeding mixtures) tested in the Czech Republic and the United Kingdom. Similarly, DON was detected in 71% of 3,420 raw and processed feed samples in Asia and the Pacific countries, although less than half of these (31%) were detected in samples from Oceania (Binder *et al.*,

2007). In the Americas, Rodrigues and Naehrer (2012) evaluated the incidence of DON in corn and soybean meal designated for animal feed and determined that 92% of 7,049 samples tested positive with levels of DON >1,000 µg/kg. In fact, 79% of these DON-positive samples tested at levels as high as 5,500 µg/kg. Additionally, the levels of DON and other trichothecenes in animal feed were highly influenced by the concentration of mycotoxins in the raw material and poor-quality controls in feed processing (Rodrigues and Naehrer, 2012).

Zachariasova *et al.* (2014) compared non-fermented versus fermented feedstuffs (wheat, barley, maize, and rye), and identified differences in the conditions that favoured the contamination of these materials. In non-fermented cereals, contamination depended on the pre-harvest and storage conditions, while water content prevailed as main condition for fermentable feed. The levels of DON detected in non-fermented ingredients varied with year whereas the fermented matrices showed consistent concentrations under the same conditions (Zachariasova *et al.*, 2014). A greater number of DON-derivatives were found contaminating fermented feed compared to non-fermented, possibly, due to the interaction between fermentation products such as carboxylic acids and alcohols with carboxyl and hydroxyl groups of DON (Zachariasova *et al.*, 2014).

Multiple factors contribute to the occurrence of DON in animal feed. Some of these could be controlled from the manufacturing process, but others are difficult to manage due to uncontrollable factors such as climate. Thus, quality control of raw material becomes crucial to reduce the worldwide occurrence of mycotoxins (DON) in animal feed and ameliorate the negative impact on animal health.

2.3.2 Adverse effects on animal performance and health

Mycotoxicosis defines the group of diseases caused by mycotoxins. Acute and chronic exposure to mycotoxin-contaminated food or feed can result in a range of deleterious effects on health and productivity. Affected animals show general symptoms that vary from gastrointestinal disorders and reproductive lesions to renal disease and chronic dermatitis (D'Mello *et al.*, 1999). The extent of the lesion and the system affected depend on the animal species and the type of mycotoxin as well as the route, time and duration of the exposure (Sobrova *et al.*, 2010). Toxic shock and death may occur if an animal is exposed to DON at extremely high concentrations;

however, these levels are rarely found in feed so chronic toxicity is the most presented clinical condition (Rotter *et al.*, 1996).

Chronic symptoms of DON mycotoxicosis are reflected in low performance parameters such as average daily gain (ADG) and feed intake (FI) as a consequence of vomiting, pain and reluctance/refusal to consume feed (Berthiller *et al.*, 2013). Swine and poultry are more sensitive to DON than large ruminants and companion animals (dogs and cats; Richard, 2007). In growing and finishing swine, low levels of DON (1,000 µg/kg) does not reduce feed consumption, but it does in piglets (Richard, 2007). At higher levels (5,000 – 20,000 µg/kg), DON induces episodes of vomiting, complete feed intake refusal, immunosuppression and renal disease (Richard, 2007). Poultry tolerate levels of DON as high as 5,000 µg/kg, but higher levels can lead to oral lesions, growth retardation, decreased egg production, poor eggshell quality, peroxidative changes in liver and immunosuppression (Murugesan *et al.*, 2015). In small ruminants such as sheep, 16,000 µg/kg of DON decreased body gain due to feed refusal (EFSA, 2014). In other domestic species such as horses, dogs and cats, chronic exposure to DON did not induce vomiting, although it reduced feed intake at concentrations of 36,000, 6,000 and 8,000 µg/kg, respectively (EFSA, 2014).

2.3.3 Regulation of DON for feed in Canada

Canadian grain is approximately 16% of the worldwide production and about 80% of it is exported. Hence, the regulation of DON levels is an important topic in terms of marketing (Qian *et al.*, 2009). In Canada, CFIA is the governmental institution responsible for setting the regulatory limits pertaining to food and feed safety. Regarding DON, from 2012–2014, the CFIA released the Food Safety Action Plan (Canadian Food Inspection Agency, 2013). This plan determined the threshold DON levels in grain-based products consumed regularly by humans and evaluated the prevalence of the mycotoxin during this period of time. The study showed that only a few samples from finished products contained low levels of DON (<1000 µg/kg) generating minor risk for human health. However, DON levels in raw materials and grain-based products had increased relative to previous years, so strategies were focussed not only in the surveillance of finished products, but also in the harvest and raw stored material conditions. Thus in 2018, the Canadian Grain Commission (CGC) started a voluntary program where grain

producers were sent a sampling kit that allowed farmers to test levels of DON in different grain crops, and therefore monitor the incidence of DON in their grain.

Currently, the CFIA in accordance with the Food and Agriculture Organization (FAO) and under the endorsement of the Guidance Document Repository, have agreed on the regulatory limits for contaminants in feed (RG-8 Regulatory Guidelines; Canadian Food Inspection Agency, 2017a). They have established the legislated maximum levels of mycotoxins (DON, T-2, HT-2, FUM, AF, ZEA, and OTA) in food, feedstuff and dairy products. For DON, the CFIA stipulated maximum levels of 2,000 µg/kg for soft wheat destined for human consumption, 5,000 µg/kg for cattle and poultry diets, and 1,000 µg/kg for swine diets, young calves and lactating animals (Canadian Food Inspection Agency, 2017a). The CFIA, with the objective of renewing the regulations for certain feed contaminants, released in July 2017 the document: Proposal – Contaminant Standards for Aflatoxins, Deoxynivalenol, Fumonisin, Ergot Alkaloids and Salmonella in Livestock Feeds (Canadian Food Inspection Agency, 2017b). This document proposes maximum limits for single ingredients and complete diets as well as the threshold for beef and dairy cattle, calves (<4 months), and lactating animals. Species that have been designated as novel production systems such as turkeys, ducks, sheep, equine and rabbits are also considered under this proposed legislation (Canadian Food Inspection Agency, 2017b). The proposed maximum levels for DON are described in Table 2.1.

2.4 Methods for control and management of *Fusarium* mycotoxins

Strategies have been developed to mitigate the negative impact of *Fusarium* spp. in the feed industries and the subsequent adverse effects on animal and human health. The objective is to reduce the levels of DON in grain, grain-based products and animal tissues. At the grain level, most of these strategies are related to crop managment such as crop rotation, fumigations and survillance. At the feed processing level, technologies have been generated to clean grain by separating out the FDK, which reduces the level of mycotoxins. Kautzman *et al.* (2015) improved grain quality by reducing the amount of FDK in grain infected with *Fusarium* using near-infrared transmittance (NIT) technology. Similarly, optical colour sorters can remove FDK from healthy (normal-coloured) kernels. Another technology of cleaning/sorting grains is the fractioning aspirator sorter, which sorts kernels according to weight through the use of an air column. With this tecnology, it is assumed that lighter kernels are affected (eg., infected with

Fusarium spp.) and therefore are separated from heavier healthy kernels. These physical methods can mechanically separate contaminated grain from healthy material thus improving grain quality in the remaining sample.

Table 2.1 Canadian Food Inspection Agency (CFIA) proposed maximum levels of deoxynivalenol (DON) for feed/total diet intended for domestic animals.

Species/Class of Animal	Single Ingredient Feeds (e.g., cereals and cereal by-products; µg/kg)	Total Diet (µg/kg)
Cattle - calves (<4 months)	5,000	1,000
Cattle - Beef	10,000	5,000
Cattle - Dairy	10,000	5,000
Lactating dairy animals	5,000	1,000
Swine	5,000	1,000
Poultry: chickens, turkeys, ducks	10,000	5,000
Sheep, equine and rabbits	10,000	5,000

Modified from: The Proposal – Contaminant Standards for Aflatoxins, Deoxynivalenol, Fumonisin, Ergot Alkaloids and Salmonella in Livestock Feeds. Available at: http://www.inspection.gc.ca/animals/feeds/consultations/contaminant-standards-for-aflatoxins-deoxynivaleno/eng/1500908795245/1500908795965

A chemical method to counteract the effect of mycotoxins is via binding the mycotoxins (e.g., DON) with a deactivating molecules or "binders" (Murugesan *et al.*, 2015). This method aims to neutralize the mycotoxin by preventing its adsorption through the cellular membrane and thus facilitating its elimination. Murugesan *et al.* (2015) described two types of binders: organic (or microbial) and inorganic. In the organic type, microbial peptides (e.g., enzymes) react with the toxins, inactivating their kinetic properties by lowering the adsorption rate. Reduction up to 25% of the adsorption rate has been observed with binders used to neutralize DON (D'Mello *et al.*, 1999). Inorganic deactivation of mycotoxins uses minerals that cannot be absorbed by intestinal cells, but can bind to the toxin therefore reducing its bioavailability in tissues (Murugesan *et al.*, 2015). However, the effectiveness of these methods is limited as they can be expensive and may interfere with the absorption of nutritional components of feed negatively affecting animal performance (Awad *et al.*, 2010). Furthermore, the products available in the market do not offer coverage for the vast number of mycotoxins that exist (Murugesan *et al.*, 2015). This concern represents an enormous problem in terms of mitigation strategies and mycotoxin control, thus challenges the industry to find novel and more efficient ways to inactivate/detoxify these harmful compounds.

2.5 Edible insects

Entomophagy, the consumption of edible insects by humans, is presumed by most to be an ancient practice from the palaeolithic era (Van Huis, 2017). Edible insects were or are part of human diets due to their natural abundance. In Africa, native comunities comsume caterpillars and termites (Illgner and Nel, 2000) while silkworms and cockroachs have been consumed in China for 3,000 years (van Huis, 2017). Even though this is usual practice for native western and eastern cultures, other parts of the world find entomophagy unrefined and improper as insects crawl in dirt and waste. However, due to intensive farming of livestock and its negative impact on the environment, newer generations are now recognizing insects as a more sustainable source of dietary protein (Varelas and Langton, 2017). With this initiative, there is a desire to portray entomophagy as a clean and healthy practice that has less impact on the environment.

The benefits of insect production are numerous. Insect rearing require a small land base and produce lower greenhouse gas emissions compared to livestock operations (Smetana *et al.*, 2016). In fact, insects are more efficient at converting feed and nitrogen into body weight than

poultry and beef species (Grau *et al.*, 2017). This is an advantage in terms of sustainability as they can be reared on waste products; for instance, Enterra Feed Corporation in Canada grows Black Soldier Fly (BSF) larvae on human food waste. These advantages are catapulting insect farming as a growth industry that could be very profitible, environmentally friendly and sustainable.

2.5.1 Yellow mealworm

Tenebrio molitor, also known as the yellow mealworm, the darkling beetle, or the flour beetle is a member of one of the largest family of beetles (Gillott, 2005). Currently, 300,000 species of the Tenebrionidae family, Order Coleoptera have been described (Van Emden, 2013). The life cycle begins with the egg. Embryonic development takes 10 to 15 days, then the first-instar larvae breaks the egg shell using its egg 'bursters', which are structures located on its head and abdomen (Van Emden, 2013). The newly hatched larvae go through 14-20 instars before it reaches the pupal stage that last between 9 and 15 days (Park *et al.*, 2014). After this period, a light brown coloured beetle emerges and within 48 -72 hours of tanning its exoskeleton, the adult turns dark brown. At this time, their sensorial organs can identify a sexual partner and they remain sexually active until their death at approximately 40 days of age (Park *et al.*, 2012). The hormone 4-methyl-1-nonanol produced by the adult females may mediate their attraction to males in the same way that Z-3-dodecenyl acetate (Z3-12:Ac) secreted from males attract females (Bryning *et al.*, 2005). When an adult female is successfully fertilized, she can lay between 200-800 eggs during her life. The life cycle takes 80.0-83.7 days, although this time can change based on different environmental settings (Park *et al.*, 2014).

Mealworm eggs can survive long periods of cold temperatures (Punzo and Mutchmor, 1980). However, eggs, larvae, pupae and beetles require favorable environmental conditions for optimal development. Descriptively, eggs are barely visible due to their millimeter size (<1 mm), with a transparent/whitish colour, elliptical shape and covered by a thin sticky layer of viscous protein (Hill, 2002). Freshly hatched larvae are soft and white with a tubular appearance. Body length in the first instar starts at about 3.5 mm and turns brown after tanning a fine hard layer of brown protective cuticle (Park *et al.*, 2014). In the molting process larvae turns from a strong hard brown body again to a soft white corpus that denotes the starting point of a new instar (14-20 instars). By the 20^{th} instar, larvae are approximately three centimeters in length, with short

antennae, small ocelli, 12 defined body segments and three pairs of legs extending from their first four segments (Van Emden, 2013). Pupae, described on occasion as 'alien shaped', are white and half-moon shaped, they wiggle when they sense any physical contact. As time approaches for the emergence of the young adult, pupae move in pulses signifying the change is eminent (Hill, 2002). The beetle emerges with active mouthparts composed of mandibles, maxillae and labium which, along with the antennae, sense the presence of feed and moisture. A defined pronotum, mesoscutellum and two striated elytra form the dorsal parts of the beetle, whereas a defined thorax, segmented abdomen and 3 pair of legs with defined tarsal segments build the ventral view (Gillott, 2005; Hill, 2002).

As poikilothermal species, all the stages are dependent on external sources of heat to survive. Punzo and Mutchmor (1980), determined that the optimal temperature and moisture conditions for the yellow mealworm are 25ºC and 75 percent relative humidity (RH). In terms of oxygen, Greenberg and Ar (1996), found an important correlation with states of normoxia (21%), and positive survivability as well as with a fast time of larvae molting.

2.5.2 Nutritional value of the yellow mealworm

According to the FAO, the world's population is expected to increase by 34% by 2050, and modern farming will not be able to generate enough food to meet the demand (FAO, 2009). As this concern grows, the food industry is challenged to find not only novel and sustainable alternatives to produce protein, but also healthy and nutritive food. *T. molitor* larvae, in their different stages, have been considered as an alternative food source due to their rich nutritional content. It was reported that crude protein (CP) ranges from 46.44-68.9%, crude fat (CF) from 31.2-32.7%, and ash from 2.86-5.7% when the larvae were fed wheat bran (Ghaly and Alkoaik, 2009; Ravzanaadii *et al.*, 2012) or organic wastes (Ramos-Elorduy *et al.*, 2002b). However, this nutritional profile may vary depending on the type and quality of the consumed diets (Nowak *et al.*, 2016). Different studies have shown changes in protein and amino acid content of insects, after they were fed diets with different levels of starch and protein (Oonincx *et al.*, 2015; Pimentel *et al.*, 2017; Van Broekhoven *et al.*, 2015). Oonincx *et al.* (2015) and Van Broekhoven *et al.* (2015) reported CP of 11.9% and CF of 2.3% after reared yellow mealworm larvae on low starch and low protein diets. Here, larval survival was higher than 87%, indicating that yellow mealworm larvae possess the ability to rapidly adapt to poor dietary conditions and yet satisfy

their maintenance requirements (Oonincx *et al.*, 2015; Van Broekhoven *et al.*, 2015). Several studies have shown that yellow mealworm larvae contain most essential amino acids (Table 2.2) and fatty acids (Table 2.3) when reared in optimal conditions. However, the presence or absence of some amino acids and fatty acids in dried yellow mealworm larvae may be also related to the richness and quality of the protein and fat source fed (Fasel *et al.*, 2017; Van Broekhoven *et al.*, 2015).

Fiber in insects is mostly contained in chitin (N-acetyl-D-glucosamine), one of the most widespread amino polymer in nature, and structurally alike to cellulose (Finke, 2007). Both polymers share strikingly similar molecular structures, except chitin contains acetamides at the C2 position of the monomers instead of hydroxyl groups on cellulose (Merzendorfer, 2006). Chitin composes the exoskeletons of arthropods, whereas cellulose strengthens the cell wall of plants (Merzendorfer, 2006). Due to these similarities, chitin (fiber) content in insects is determined in the acid detergent fiber (ADF) portion, which is normally used to detect levels of cellulose in plants. Additionally, ash and the acid detergent insoluble nitrogen (ADIP) associated with some other structural amino acid may form part of the whole ADF portion in insects (Finke, 2015). Marono *et al.* (2015) determined ADF, ADIP and chitin content in yellow mealworm meal and reported values that ranged from 7.52-11.4%, 2.72-5.10%, and 4.80-6.73% respectively.

Due to chitin's chemical nature, its content in mealworm meal could affect protein digestibility in omnivorous species, as reported in Nile tilapia (Sánchez-Muros *et al.*, 2016). Carnivores in comparison, have a more optimal complement of enzymes such a gastric chitinase, chitobiase and lysozymes to aid the breakdown of up to 5% (dry matter) of chitin without affecting growth performance (Karlsen *et al.*, 2017). Furthermore, yellow mealworm larvae chitin has shown positive effects in broiler chicks by stimulating the production of immunoglobulin G and A and reducing ceca *E. coli* and *Salmonella* spp. (Islam and Yang, 2017).

Table 2.2 Amino acid profile of yellow mealworm larvae fed different diets from four studies (dry matter percent).

Amino acid %	**Studies**		
	Ravzanaadii *et al.*, (2012)[1]	**Ramos-Elorduy *et al.*, (2002)[2]**	**Ghaly and Alkoaik, (2009)[3]**
Threonine	1.81	3.63	2.1
Methionine	0.67	1.06	2.0
Valine	2.44	5.86	6.4
Isoleucine	3.56	4.03	4.8
Leucine	3.41	6.91	8.2
Phenylalanine	1.76	6.66	4.6
Lysine	2.90	4.65	5.3
Histidine	1.53	2.89	1.7
Arginine	2.43	4.24	4.3
Tyrosine	3.46	ND	4.0
Cysteine	0.52	1.00	5.6
Glutamic acid	5.68	11.02	12.3
Proline	3.02	NA	NA
Glycine	2.41	5.19	2.0
Aspartic acid	3.60	7.23	NM
Alanine	3.69	7.73	6.8
Serine	2.09	4.25	2.6
Crude protein (%)	46.4	47.7	63.3

NA: no analyzed

ND: undetected

[1] Diet based on wheat bran

[2] Diet based on organic wastes and yeast

[3] Diet based on wheat flour, wheat bran and oat meal.

Table 2.3 Fatty acid composition of yellow mealworm larvae fed different diets from four studies (dry matter percent).

Fatty Acid			**Studies**		
Common name	**Lipid number**	**ω-n**	**Ravzanaadii *et al.*, (2012)[1]**	**Finke, (2015)[2]**	**Van Broekhoven *et al.*, (2015)[2]**
Myristic acid	(C14:0)		3.05	1.43	-
Palmitic acid	(C16:0)		16.72	12.30	16.96
Stearic acid	(C18:0)		2.49	2.56	2.72
Palmitoleic acid	(C16:l)	**ω-7**	2.67	0.84	2.88
Oleic acid	(C18:l)	**ω-9**	43.17	27.30	46.41
Vaccenic acid	(C18:l)	**ω-7**	0.03	-	0.39
Eicosenoic acid	(C20:l)	**ω-9**	0.24	0.19	-
Linoleic acid	(C18:2)	**ω-6**	30.23	24.3	27.83
γ-Linoleic acid	(C18:3)	**ω-6**	0.05	<0.07	-
Linolenic acid	(C18:3)	**ω-3**	1.36	1.03	1.48
Arachidonic acid	(C20:4)	**ω-6**	-	<0.07	-
Eicosapentaenoic acid	(C20:5)	**ω-3**	-	0.22	-
Docosatetraenoic acid	(C22:4)	**ω-6**	-	-	-
Docosahexaenoic acid	(C22:6)	**ω-3**	-	<0.07	-
Crude fat (%)			32.7	34.4	33.4

[1] Diet based on wheat bran

[2] It does not specify diet source

- Undetected

2.5.3 *Tenebrio molitor* fed wheat contaminated with mycotoxins

Recent studies have evaluated production traits and breeding parameters in yellow mealworm larvae exposed to wheat infected with *Fusarium* spp. (Guo *et al.*, 2014), and contaminated with mycotoxins DON (Guo *et al.*, 2014; Van Broekhoven *et al.*, 2017), ZEA, OTA, and T-2 (Van Broekhoven *et al.*, 2014). Increased mortality were observed in larvae fed kernels infected with *F. bassiana* and *F. culmorum* containing 10,240 µg/kg DON (Guo *et al.*, 2014); however, mortality was not affected when *T. molitor* were fed either 8,000 µg/kg of DON spiked-diet (Van Broekhoven *et al.*, 2017) or >500 µg/kg of ZEA, OTA and T-2 (Van Broekhoven *et al.*, 2014). In fact, under these circumstances, the yellow mealworm larvae had increased body weight, and undetectable levels of DON in larvae body. Additionally, Van Broekhoven *et al.* (2017) reported that 41% of ingested DON from the DON-spiked diet (8,000 µg/kg) and 14% from the naturally occurring diet (4,900 µg/kg) was excreted in feces collected from larvae after 24 hours of fasting. On the other hand, lower levels of ZEA, OTA and T-2 were detected in larvae tested directly after feeding them the contaminated diet. Although ZEA and OTA concentrations decreased to non-detectable levels in larvae tested after 24 hours of fasting, low levels of T-2 were still detectable at 72 hours of fasting (Van Broekhoven *et a*l. 2014).

The mechanism of how mealworms detoxify or biotransform these molecules is not well understood; however, it appears that DON is being metabolized and/or partially excreted through frass (Van Broekhoven *et al.*, 2017). A study performed by Wolfarth *et al.* (2015) suggested that natural soil fauna such as the arthropod *Folsomia candida* in association with a soil nematode *Aphelenchoides saprophilus* accelerated the degradation of DON from *Fusarium*-infected wheat straw under field conditions via microbial breakdown. The enzymes amylase, cellulase, laminarinase, chitinase, licheninase, and β-glucosidase have demonstrated catalytic effects on DON and other mycotoxins produced by *Fusarium* (Genta *et al.*, 2006). These enzymes are not only produced by the microbiota but also in yellow mealworm larvae midgut, and possibly co-operate together in the detoxification of DON (Genta *et al.*, 2006). It has been suggested that cytochrome P450 monooxygenases (P450s) in insects are important for oxidation-reduction reactions that aid in the detoxification of a wide range of toxins (Scott and Wen, 2001). However, enzymatic activity may not be the only pathway for the metabolism of DON in the yellow mealworm larvae, but perhaps is the combined interaction of the gut microbial enzymes with the larval midgut and the fat body activity.

2.5.4 Insects in animal feed and current world production.

To date, entomophagy is more prevalent in human communities. A clear example of this is in the Netherlands where some insect species are available and under regulation in markets for consumption (Janssen *et al.*, 2017). Surprisingly, however, the market for insect-based protein for animal feed is only just in its infancy and research is underway, fueled by the urgent need for alternative sources of protein and regulation for this market. Recently, many companies (>2,000) have been formed worldwide to research and commercialize insect-based feed ingredients for human and animal consumption. In December 2016, the South-African company AgriProtein, along with the Austrialian partner Twynam, announced the production of insect-based protein as a replacement for fishmeal in aquaculture (Byrne, 2016). In May 2017, the European Union Regulation 893 accepted the implementation of meals from insect species such as the BSF (*Hermetia illucens*), common housefly (*Musca domestica*), the yellow mealworm, the lesser mealworm (*Alphitobius diaperinus*) and crickets (*Acheta domesticus, Gryllodes sigillatus* and *Gryllus assimilis*) for fish feed, which boosted the insect market for aquafeed in Europe (European Union, 2017). Research has focused on demonstrating the beneficial effects of insect-meals in the performance and health of commercial aquatic species including white leg shrimp (*Litopenaeus vannamei*), rainbow trout (*Oncorhynchus mykiss*), European seabass (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*; Karlsen *et al.*, 2017; Sánchez-Muros *et al.*, 2016; Wang and Chen, 2005). New research is needed in order to expand the use of the insect protein to other commercial livestock species such as poultry and swine. Recently, after approval of insect meals in the EU for animal feed, the Aarhus University in Denmark was granted €2.55 million to start 'InVALUABLE', large-scale production of mealworms destined for the aquaculture and poultry industry (Koeleman, 2017). In North-America, the Canadian company Enterra Feed Corporation was granted approval by the CFIA to produce BSF meal for aquaculture and poultry nutrition, and has received approval to sell insect-based products to the USA and EU (Leung, 2017). By 2020, insect meal is expected to be approved for poultry and swine species in North America and EU, which will allow animal producers to replace up to 10% of the conventional sources of feed protein with insect-derived ingredients (Koeleman, 2016). These changes will also contribute to the development of the insect sector, create more market and employment opportunities as well as lower the cost of animal production.

2.6 Hypothesis and Objective

The overall objective of this thesis research was to determine whether yellow mealworm larvae could be cultivated on mycotoxin-contaminated wheat to produce a safe, alternative source of protein for animal feed. To achieve this objective, yellow mealworm larvae were reared on *Fusarium*-infected wheat containing high levels of DON, and accumulation of DON in larvae body along with effects of DON exposure on production, survival, nutritional traits and preference behaviour of larvae were measured.

The specific hypotheses of this thesis research were:

- The yellow mealworm larvae will not accumulate high levels of DON from *Fusarium* damaged wheat and it will provide an alternative safe protein ingredient for animal feed.
- Rearing larvae on wheat contaminated with DON at levels as high as 12,000 µg/kg will not affect insect production traits.
- Feeding larvae on wheat contaminated with DON at levels as high as 12,000 µg/kg will have not affect on larval nutritional profile.
- The yellow mealworm larval nutritional profile will not be affected by either freeze-dried or oven-dried method.
- The yellow mealworm will show a feeding preference for wheat kernels infected with *Fusarium graminearum* and contaminated with DON compared to uninfected kernels.

Preface to chapter 3

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Author contributions:

C. Ochoa-Sanabria contributed to the experimental design, executed the experiment, compiled and analysed data, and wrote the manuscript.

N.S. Hogan contributed to the experimental design, interpretation of toxicological data and commented and edited the manuscript.

K.M. Madder started and maintained the colony, contributed to the experimental design and commented and edited the manuscript.

C. Gillott provided guidance on the maintenance of the colony and edited the manuscript.

B.R. Blakley optimised the mycotoxin analysis on dried mealworms and commented on the manuscript.

M. Reaney performed the fatty acid profile method on the dried mealworm samples and commented on the manuscript.

A.D. Beattie identified the species of *Fusarium* colonizing our wheat samples and commented and edited the manuscript.

F.C. Buchanan provided scientific input and guidance throughout all the stages of this research and commented and edited the manuscript as well as obtained the funding to perform the research.

CHAPTER 3

3. YELLOW MEALWORM LARVAE (*TENEBRIO MOLITOR*) FED MYCOTOXIN-CONTAMINATED WHEAT – A POSSIBLE SAFE, SUSTAINABLE PROTEIN SOURCE FOR ANIMAL FEED?

3.1 Introduction

Fusarium mycotoxins in food and feed are a recurring problem for crop and animal production worldwide. Several *Fusarium* species are widespread fungal pathogens affecting cereals and stored grains and are causative agents of FHB disease in wheat (Gräfenhan *et al.*, 2013). *Fusarium* produces secondary metabolites, also known as mycotoxins, which can downgrade the nutritive, physical, and chemical qualities of grain due to the presence of FDK (Dill-Macky and Jones, 2000). In 2014, it was estimated that two million metric tonnes of wheat and durum were downgraded to salvage (greater than 10% FDK) costing 600 million dollars to Canada's economy. Two years later, approximately one billion dollars in losses were associated with widespread *Fusarium* infection in Western Canadian wheat crops (Canadian Grain Comission, 2016).

Fusarium graminearum and *F. culmorum* are the two most prevalent *Fusarium* species in the Canadian prairie provinces and are considered the most harmful due to their ability to produce multiple mycotoxins, primarily DON (Gräfenhan *et al.*, 2013). In years with high *Fusarium* infection, farmers are challenged to sell their grain at a lower profit if it exceeds the DON regulatory limit established by the CFIA. Consequently, grain with significant amounts of FDK is either blended with uncontaminated or low FDK grain, or condemned to salvage grain with no commercial value.

Several strategies have been developed to mitigate the negative impact of mycotoxins in the feed industry. For example, decreasing the amount of FDK via cleaning or neutralizing mycotoxins to improve the grain quality can reduce the amount of salvage grain and improve the commercial value. Near-infrared transmittance technology, for instance, is capable of separating out individual FDK to improve the quality and value of *Fusarium* downgraded grain (Kautzman *et al.*, 2015). Alternatively, mycotoxin binders can be incorporated into feed to adsorb and deactivate certain mycotoxins, preventing uptake and toxicity in animals (Karlovsky, 2011). However, the lack of highly effective disease controls measures, such as genetic resistance or fungicides, and the large impact environmental conditions have on the development of FHB, makes this disease extremely difficult to control. Industry is therefore challenged to find novel and more efficient ways to inactivate/detoxify these harmful compounds as well as to reduce economic losses to the grain sector.

Historically, insects have been an important part of the human diet. Entomophagy, the consumption of edible insects by humans, was an ancient practice from the palaeolithic era, but is still currently practiced by many ethnic comunities worldwide (Van Huis, 2017). Edible insects contain high nutritional value and are critical to supply the world's growing population, even when reared on organic wastes (Ramos-Elorduy *et al.*, 2002b). For example, yellow mealworm larvae (*Tenebrio molitor*) have the capacity to recycle low value organic materials and convert them into a source of protein. The nutritional value of larvae fed waste material was reported as 47.7% CP, 37.7% CF, 5% crude fiber and 2.9% ash (Ramos-Elorduy *et al.*, 2002b; Ravzanaadii *et al.*, 2012).

A recent study by Guo *et al.* (2014) demonstrated that yellow mealworm larvae preferred wheat kernels artificially infected with *Fusarium proliferatum, F. poae* and *F.culmorum* over uninfected kernels. These individuals had increased body weight with undetectable levels of DON and ZEA; however, there was high mortality in larvae fed *F.culmorum* (Guo *et al.*, 2014). Van Broekhoven *et al*. (2017) reported no effect on survival when yellow mealworm larvae consumed concentrations of DON from either a DON-spiked (8,000 µg/kg), or a natural *Fusarium*-infected wheat source (4,900 µg/kg DON); however, DON was excreted in frass at a proportion of 41% and 14%, respectively. These findings suggest that yellow mealworm larvae may detoxify or biotransform DON into derivative forms or unknown metabolites. Based on these initial reports, there is potential for yellow mealworm larvae to be cultivated on mycotoxin-contaminated wheat to produce a safe, alternative source of protein for animal feed. The goals of this study were to determine the potential accumulation of DON in yellow mealworm larvae reared on *Fusarium*-infected wheat containing high levels of DON, and investigate the effects of DON exposure on production, survival and nutritional traits of larvae.

3.2 Materials and methods

3.2.1 Wheat sources

Two sources of the Canada Western Red Spring wheat were purchased from grain producers in Saskatchewan, Canada. Mycotoxin concentration was determined using HPLC-tandem MS at Prairie Diagnostic Services (Saskatoon, Canada). One wheat source was found to initially contain 0 µg/kg and was subsequently used as the control wheat. The other wheat source

contained DON at 8,000 µg/kg. The 8,000 µg/kg DON wheat was sorted using a BoMill IQ NIT grain sorter at Flaman Seed Cleaning and Handling Facility (Saskatoon, Canada) to concentrate the DON levels. This resulted in six quality-sorted fractions, from low to high quality with increasing levels of DON. These were re-analysed for DON levels (data not shown) and fractions one and two, three and four and five and six were combined to obtain three different concentrations of DON for the experimental diets: low, medium and high. The three diets and the control wheat were ground to ~1000-micron particle size using a hammer mill HMS.20X (Canyon City, USA) and retested for mycotoxins. The final DON levels fed to mealworms were: 200 µg/kg (control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high). Additionally, analysis of dietary CP using Dumas-combustion method using a Leco FP-528 (St Joseph, USA) revealed CP levels of 15.0%, 11.1%, 10.5% and 10.7% for control, low, medium, and high diets, respectively.

3.2.2 *Fusarium* species identification

Wheat kernels were surface-sterilized in 3% (v/v) NaOCl for 2 min and rinsed twice with water. One hundred kernels were randomly selected and plated on potato dextrose agar with 2 mg/L each of streptomycin, tetracycline and neomycin in petri dishes (10 seeds per dish). After 5-7 days at 25°C under near UV light, *Fusarium* colonies were isolated and identified based on morphological characteristics (Gerlach and Nirenberg, 1982). Samples of frass (purified and a mixture with wheat crumbs/fines) were also cultured for *Fusarium* spp. to determine if the fungus could survive passage through the yellow mealworm larvae gastrointestinal tract. Samples were diluted to 1% and 2% (g/mL) with sterile water and 0.3 mL of each dilution was spread on potato dextrose agar surface. After seven days culturing at 25°C under near UV light, fungal colonies were identified by examining morphological characteristics (Gerlach and Nirenberg, 1982).

3.2.3 Yellow mealworm colony

Five hundred larvae and 50 beetles of *T. molitor* were purchased from Carolina Biological Supply (Burlington, USA). Larvae and beetles were reared in large plastic containers (50 x 32 x 15 cm) with ground wheat (0 µg/kg DON) as the only source of food. Paper towels were laid on top of the wheat and spritzed with water three times per week. The rooms were maintained at

50% RH, 25 °C and a 12-hour light photoperiod. Pupae were moved from the main colony into a separate plastic container twice weekly. Once beetles emerged, they were placed into a new container.

3.2.4 Feeding trial

A total of 6,000 mealworms (7^{th} to 9^{th} instar; Park *et al.*, 2014) were selected from the mealworm colony using grain cleaning 5.0 slotted mesh (1.98 x 19.05 mm). Three hundred mealworms were allocated per replicate and placed in plastic drawers (22.5 x 16.5 x 9 cm) containing 500 g of ground diet along with a paper towel laid on top. Each treatment was replicated five times.

Larvae were reared on one of the four diets until the first two pupae were observed in a replicate. The number of days to reach this endpoint, the pooled larval weight (minus any mortality) and the weight of the remaining feed was recorded to calculate feed intake (FI), average daily gain (ADG), feed conversion ratio (FCR) and efficiency of conversion of ingested food (ECI) according to the formulas below. Survivability was assessed by daily counting of dead mealworms. After collecting the final weights, larvae were placed in an empty container to fast for 24 hours and then washed with distilled water. The frass was collected and refrigerated at 4^{o}C and the larvae were euthanized by freezing at -20°C. Protein efficiency ratio (PER) was calculated to determine protein quality of the diets and the efficiency of the larvae in converting protein from DON-contaminated wheat into body weight.

- ADG = (Final weight - initial weight)/number of days on feed
- FCR = Weight of ingested feed/weight gained
- ECI = (Weight gained/weight of ingested feed) x 100%
- PER = Weight gained/total protein fed (dry basis)

3.2.5 Breeding trial

The breeding trial was a similar arrangement as the feeding trial except there were 200 mealworms per replicate, thus totalling 4,000. Production data collected from larvae exposed to the four levels of DON included the number of days until larvae reached the endpoint, the development time required for 100 (50%) larvae to pupate and the average weight of the new

pupae and new beetles. Survival was monitored in larvae, pupae and beetles by daily counting of dead insects. A second generation of larvae was generated (per four treatments) to observe the effect of mycotoxins on body weight and survivablity in the offspring. Insects were moved to larger plastic containers and 500 g of additional diet was added. Upon accumulating 50 pupae, the weight of the pupae was recorded, along with the weight of 200 larvae and 100 beetles.

Replicates one and two from both the feeding and breeding trials (see below) were carried out concurrently in October 2016, while replicates three through five were conducted in November 2016. Room conditions and watering regimes during these trials were the same as those used to maintain the colony.

3.2.6 Preference trial

To measure avoidance or attraction of mealworms to mycotoxin-contaminated diets, thirty naive larvae were randomly selected from the main colony and allocated ten per replicate (n=3). Each petri-dish (replicate) was divided into four quadrants. Each quadrant containing 20 g of one of the four diets (control, low, medium and high). Each diet was placed at the outside edge of the petri dish within the quadrant. Thereafter, the ten mealworms were randomly selected and positioned in the centre of the petri-dish and left in a dark container without any disturbance. Thirty minutes later, the worms within each sector were counted. This process was repeated 20 times.

3.2.7 Mycotoxin analysis

Mycotoxin concentration was tested in wheat, freeze dried larvae (FDL) and frass. Samples were analysed for 14 common mycotoxins at Prairie Diagnostic Services Inc., (Saskatoon, Canada) via ultra-high-performance liquid chromatography Agilent 1100, (Santa Clara, USA) and mass spectrometry Micromass Quattro Ultima Platinum Mass Spectrometer, (Milford, USA). The mycotoxin suite included DON and metabolites 3-ADON and 15-ADON, NIV, ZEA, α-zearalenol, β-zearalenol, T-2, HT-2, diacetoxyscirpenol, OTA, and aflatoxin B1 (AFB_1). Mycotoxin testing in larvae was analysed per replicate while frass was assessed per treatment using a pooled sample from all replicates.

3.2.8 Chemical nutritional analysis of larvae

The moisture content of the larvae fed the four diets was determined after freeze-drying using a Labconco FreeZone 6 Liter Benchtop Freeze Dry System (Kansas City, USA). An extra sample of larvae fed the control diet was oven-dried (AOAC, 1990; method 930.15) to determine any differences in nutritional value between oven-dried larvae (ODL) and FDL. The ODL sample was dried at 60°C overnight, then dry matter was analysed (AOAC, 1990; method 930.15). To determine ash content, 2.2 g of dried sample (pooled from replicates) was ignited at 600 °C for 18 hours (AOAC, 2012; method 942.05). To analyse ADF, duplicates 0.5 g samples ground to a particle size of 1 mm were evaluated per treatment using an ANKOM^{200} fiber analyser (AOAC, 1977; method 973.18). The remaining portion of the samples were used to estimate crude protein and obtain acid detergent insoluble nitrogen (ADIN; Marono *et al.*, 2015). The chitin percentage was calculated from the ADF ash-free and ADIN values as described by Marono *et al*. (2015). Neutral detergent fiber (NDF) was not assessed.

Crude protein was determined by two methods. For the Dumas-combustion method, 0.2 g of 1 mm ground dried larvae was placed in a gel capsule and combusted at 800°C following the guideline of AOAC, 1997; method 990.03. Kjeldahl CP analysis (AOAC, 1994; method 984.13) was performed as part of the ADIN analysis used for chitin estimation. The N conversion factor was 6.25 for both Dumas-combustion and Kjeldahl digestion methods. The amino-acid profile of larvae was determined by the nutrition lab of the Faculty of Agricultural and Food Sciences at University of Manitoba, (Winnipeg, Canada; AOAC, 1995; method 994.12) using amino acid analyser S2100 Sykam, (Eresing, Germany). To assess PER, a sample of each diet was analysed for CP by Dumas combustion and calculated as outlined in Gasco *et al*. (2016).

Crude fat was assessed by the ethyl ether extraction gravimetric method (AOAC, 2000; method 920.39) using a Goldfisch extraction apparatus model 3500 (Kansas City, USA). Larvae samples of 1.3 g were processed in duplicates per treatment and extracted for 16 hours. For the fatty acid (FA) profile, lipids from the larvae samples (25 mg) were extracted and trans-esterified with 500 µL of 0.5 N alcoholic NaOH solution under nitrogen at 100ºC for 10 min. Residual fatty acids were then esterified by addition of 500 µL of BF_3/methanol reagent under nitrogen at 100ºC for 30 min. After cooling to 30-40^{o}C, 2 ml of hexane was added and mixed. Saturated NaCl was then added and the layers were mixed and allowed to separate. The upper layer with fatty acid methyl esters (1 microL) was injected on an Agilent 6850 gas chromatograph

complemented with an Agilent 7683 series autoinjector (Santa Monica, USA) Fatty acids were detected by flame ionization after separation on a DB-17 column (30 m x 0.32 mm x 0.25 mm film thickness). Hydrogen was used as a carrier gas (4.3 mL/min; split ratio = 70:1) while nitrogen (40 mL/min) was used as the makeup gas. The detector temperature was 280 ºC. The initial oven temperature (130 ºC) was increased ºC/min to 175 ºC then 60ºC/min to 280ºC after which it was held for 1.25 min. The total run time was 12 min per injection.

3.2.9 Statistical analysis

Feeding and breeding trial data were analyzed as a randomized complete block design (RCBD). The statistical model used was: $Y_{ij} = \mu + P_i + \alpha_j + e_{ij}$, where, Y_{ij} was the dependent variable associated with block i and treatment j; μ was the overall mean; P_i was the block effect associated with the two different time periods over which the trial was conducted; α_j was the fixed effect associated with the four diet treatments; and e_{ij} was the random error associated with observation ij.

The preference trial was assessed as a completely randomized design (CRD) following the model: $Y_{ij} = \mu + \alpha_j + e_{ij}$, where model parameters were the same as described for the RCBD experiments. All experiments were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Data were normally distributed when analyzed by the Shapiro-Wilk test. Comparison of treatment means was assessed using the Tukey-Kramer HSD test at a $P<0.05$ level of significance. Data obtained using pooled replicates were not statistically analyzed, but the values are reported.

3.3 Results

3.3.1 Mycotoxin levels

Larvae fed four diets differing in the concentration of DON were analysed for multiple mycotoxins. The mycotoxins DON, 3-ADON and NIV were the only mycotoxins detected in dried larvae and frass (Table 3.1). There was no difference in the level of DON detected in dried larvae between diets (P=0.883). Similarly, 3-ADON in larvae did not differ numerically among the treatments although it should be noted that it was only detected in one replicate of larvae from each of the medium and high treatments. The excretion of DON, 3-ADON and NIV was measured in a pooled sample of frass. Levels in the frass were higher when compared to levels in

the dried larvae within the low, medium and high diets (Table 3.1). Similarly, the 3-ADON levels detected in frass were higher than that detected in the dried larvae, including the control diet. Low levels of NIV were identified in frass from larvae fed the control and medium diets. *Fusarium graminearum* was identified as the species present in the wheat source used to formulate the low, medium and high diets, but no *Fusarium* species were isolated from the frass or the frass wheat crumb mixture.

Table 3.1 Levels of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and nivalenol (NIV) detected in the diets, dried mealworms, and frass (µg/kg) of yellow mealworm larvae fed four different levels of DON-contaminated wheat.

Mycotoxin	**Treatments**	**Diets**	**Mealworm[1]**	**Frass[2]**
DON	Control	210	136.4±40.5	131.0
	Low	2,100	127.2±30.5	324.0
	Medium	10,000	121.8±19.3	230.4
	High	12,000	131.0±23.6	742.1
3-ADON	Control	<LOD	66.2±0.1	285.6
	Low	62.8	65.6±0.2	322.6
	Medium	52.2	65.5[*]	326.4
	High	204.9	65.3[*]	279.5
NIV	Control	< LOD	< LOD	49.5
	Low	< LOD	< LOD	< LOD
	Medium	< LOD	< LOD	50.9
	High	< LOD	< LOD	< LOD

[1]Mean of replicates (n=5) per treatment ± standard deviation

[2]Pooled samples from five replicates

[*]Detectable only in one replicate

<LOD: Below limit of detection

Limit of Detection for DON, 3-ADON and NIV was 25.0 µg/kg

3.3.2 Feeding trial and yield performance

In the feeding trial, survivability was higher than 96%, the length of time to reach the experimental endpoint ranged from 32.2±4.0 to 34±2.7 days and the ADG per worm ranged from 1.9±0.1 to 2.1±0.1 mg/day (Table 3.2). There was no effect of diet on any of these parameters ($P>0.05$). No differences were observed with PER ($P=0.050$). The FCR differed between the control (2.1±0.2) and low (2.9±0.40) treatment ($P=0.008$), and ECI in mealworms fed the control (47.3±6.0%) was higher than those on the low (35.4±4.8%) or medium (38.0±4.1) diets ($P=0.005$).

Table 3.2 Production parameters, performance and survival of yellow mealworm larvae fed four different levels of deoxynivalenol (DON)-contaminated wheat.

	Treatments[1]				SEM	P value
Parameter	**Control**	**Low**	**Medium**	**High**		
Days to endpoint	32.2±4.0	33.6±3.3	34.0±2.7	34.0±2.7	1.450	0.791
Survival (%)[2]	96.4±2.5	97.7±0.8	96.7±1.7	98.0±1.9	2.434	0.471
Larval weight (mg)[3]	93.3±8.4	99.9±9.8	101.9±7.4	100.2±10.4	4.053	0.474
ADG (mg/day)[3]	1.9±0.1	2.1±0.1	2.1±0.1	2.0±0.1	0.044	0.169
FCR [3]	$2.1\pm0.3_{a}$	$2.9\pm0.4_{b}$	$2.7\pm0.3_{ab}$	$2.5\pm0.1_{ab}$	0.129	0.008
ECI (%)[3]	$47.3\pm6.0_{a}$	$35.4\pm4.8_{b}$	$38.0\pm4.1_{b}$	$40.0\pm2.0_{ab}$	2.007	0.005
PER[3]	3.1±0.4	3.2±0.4	3.6±0.4	3.7±0.2	0.165	0.050

[1]Mean of replicates (n=5) per treatment ± standard deviation

[2]Trait evaluated per 300 larvae

[3]Traits evaluated per individual larvae

Values within a row that are followed by a different letter indicates significant differences ($P<0.05$)

Abbreviations: ADG (average daily gain); FCR (feed conversion ratio); ECI (Efficiency of conversion of ingested food); PER (protein efficiency ratio)

Concentration of DON in diets: 200 µg/kg (control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

3.3.3 Chemical nutritional analysis

The chemical nutritional composition of larvae reared on each of the four DON-contaminated diets plus the oven-dried control sample is reported in Table 3.3. The ODL and FDL values were similar for the control diets. Larval CP values ranged from 49.44 to 51.85%. Crude fat ranged from 34.24 to 35.97%. Ash was similar across the treatments. In terms of fiber, ADF and ADIN were numerically higher for ODL, though chitin values were similar between ODL and FDL samples. Four FA were detected (Table 3.4). Oleic acid (C18:1) was the most abundant with values between 55.00 to 56.86% of the total FA content. Linoleic acid (C18:2) had values ranging between 17.47 to 21.53%. Palmitic acid (C16:0) had slightly lower levels that ranged from 16.94 to 20.41%, whereas myristic acid (C14:0) was the least abundant FA detected, ranging from 5.18 to 6.98%. The ODL samples contained more myristic and palmitic acid than FDL but were lower in linoleic acid. The FA palmitoleic acid (C16:l), stearic acid (C18:0), vaccenic acid (C18:l) γ-linoleic acid (C18:3), linolenic acid (C18:3), eicosenoic acid (C20: l), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5), docosatetraenoic acid (C22:4), docosahexaenoic acid (C22:6) were analysed but undetected in our samples. The amino acids detected in the larvae are reported in Table 3.5. Overall, amino acid values were the same among the treatments (including ODL), except for cysteine where a lower amount was observed in ODL compared to FDL.

Table 3.3 Chemical nutritional analysis of yellow mealworm larvae fed different four different levels of deoxynivalenol (DON)-contaminated wheat (dry matter percent).

Parameter	**Treatments**				
	ODL	**FDL**			
	Control	**Control**	**Low**	**Medium**	**High**
Dry Matter	39.70	39.89	40.99	42.37	40.10
Crude Protein	50.15	51.85	49.44	50.65	49.56
Crude Fat	34.35	34.74	35.97	35.47	35.24
Ash	3.23	3.64	3.41	3.39	3.45
ADF	5.74	5.30	5.23	5.57	5.36
ADIN	2.95	2.60	2.59	2.73	2.64
Chitin	2.79	2.70	2.65	2.85	2.72

Abbreviations: ODL (oven-dried larvae), FDL (freeze-dried larvae), ADF (acid detergent fiber), ADIN (acid detergent fiber insoluble nitrogen)

Concentration of DON in treatments: 200 µg/kg (ODL fed control), 200 µg/kg (FDL fed control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

Table 3.4 Fatty acid profile of yellow mealworm larvae fed four different levels of deoxynivalenol (DON)-contaminated wheat (dry matter percent).

Fatty Acid			**Treatments**				
			ODL	**FDL**			
Common name	**Lipid number**	**ω-n**	**Control**	**Control**	**Low**	**Medium**	**High**
Myristic acid	C14:0		6.98	5.60	5.18	5.28	5.28
Palmitic acid	C16:0		20.41	16.94	18.05	18.18	18.61
Oleic acid	C18: l	ω-9	55.13	56.86	55.47	55.00	55.28
Linoleic acid	C18:2	ω-6	17.47	20.59	21.30	21.53	20.82

Abbreviations: ODL (oven-dried larvae), FDL (freeze-dried larvae)

Concentration of DON in treatments: 200 µg/kg (ODL fed control), 200 µg/kg (FDL fed control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

Table 3.5 Amino acid content of yellow mealworm larvae fed four different levels of deoxynivalenol (DON)-contaminated wheat (dry matter percent).

Amino acid	**Treatments**				
	ODL	**FDL**			
	Control	**Control**	**Low**	**Medium**	**High**
Aspartic Acid	4.205	4.293	4.108	4.200	4.257
Threonine	2.084	2.129	2.059	2.073	2.117
Serine	2.610	2.677	2.564	2.580	2.670
Glutamic Acid	6.188	6.037	5.811	5.918	5.954
Proline	3.289	3.433	3.301	3.405	3.388
Glycine	2.742	2.821	2.790	2.787	2.840
Alanine	4.033	4.094	4.053	4.054	4.145
Cysteine	0.381	0.493	0.450	0.458	0.485
Valine	2.950	2.917	3.014	2.992	2.964
Methionine	0.633	0.698	0.605	0.646	0.695
Isoleucine	2.012	2.010	2.101	2.101	2.059
Leucine	3.654	3.711	3.651	3.704	3.726
Tyrosine	4.033	3.757	3.748	3.846	3.900
Phenylalanine	1.920	1.855	1.801	1.853	1.861
Histidine	4.834	4.954	4.797	4.818	5.111
Lysine	2.557	2.718	2.711	2.763	2.717
Arginine	2.628	2.710	2.736	2.720	2.732
NH_3	0.700	0.664	0.713	0.716	0.677

Abbreviations: ODL (oven-dried larvae), FDL (freeze-dried larvae)

Concentration of DON in treatments: 200 µg/kg (ODL fed control), 200 µg/kg (FDL fed control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

3.3.4 Breeding and preference trials

In the first generation, survival to the pupal stage for larvae fed the control diet was higher (99.6±0.6%) compared with larvae fed the low (98.2±0.7%), medium (96.9±0.9%) and high (98.4±0.9%; P=0.001; Appendix A) DON-contaminated diets. The mortality observed from larvae to adult stage did not exceed 3.5% for any of the diets. Adult weights in the first-generation were 103.8±8.1 mg for the control, and 101.5±4.4 mg, 102.3±5.4 mg, and 105.4±4.3 mg for low, medium and high, respectively. In the second-generation, beetle weight differed between diets (P=0.001; Appendix B). The average weight of 100 beetles was higher on the control (88.0±4.3 mg) compared to the DON-contaminated diets low, medium and high (77.8±1.5, 80.2±3.3 and 76.0±3.2 mg respectively). Adults from the second generation weighed less than those observed in the first (Appendix B). There were no differences between diets in terms of preference or avoidance (P=0.074; Appendix C).

3.4 Discussion and conclusion

3.4.1 Mycotoxin analysis

A major objective of this study was to determine the potential accumulation of mycotoxins in yellow mealworm larvae reared on *Fusarium*-damaged wheat with DON concentrations as high as 12,000 µg/kg. Our results demonstrated that after consuming high concentrations of DON for four weeks, DON was detected in the larvae, but did not exceed 136 µg/kg. Deoxynivalenol was not detected in larvae fed *F. culmorum*-infected wheat with 10,240 µg/kg of DON (Guo *et al.*, 2014) nor in directly harvested or fasted larvae fed 4,900 µg/kg (naturally contaminated) and 8,000 µg/kg (spiked) DON (Van Broekhoven *et al.*, 2017). The acetylated derivative 3-ADON was detected in concentrations up to 66.2±0.10 µg/kg in larvae but this was not consistent among all the replicates. Camenzuli *et al.* (2018) reported undetectable levels of 3-ADON in *Alphitobius diaperinus* and *Hermetia illucens* larvae fed diets spiked with DON, ZEA, AFB_1 and OTA. Similarly, Van Broekhoven *et al.* (2017) did not detect DON derivatives (15-ADON and DON-3G) in yellow mealworms fed diets containing these metabolites. In our study, DON and 3-ADON may have been detected due to partial metabolism of the high doses of DON and the conversion of it into 3-ADON via acetylation. Additionally, we exposed the larvae to higher levels of DON in naturally contaminated wheat and conducted a longer exposure (33.6±3.29 days) than the 15 days reported in the Guo *et al.* (2014) and Van Broekhoven *et al.*

(2017) trials. Another reason for the detection of DON and 3-ADON may be due to a specific interaction between the larvae and the species of *Fusarium (F. graminearum)* contained in our diets as Guo *et al.* (2014) noted that yellow mealworm larvae reacted differently to other species of *Fusarium.* Together, these factors could have facilitated the sequestration and therefore detection of DON and 3-ADON in the larvae.

In our samples, DON was partly excreted through faeces. The concentration of DON excreted by larvae increased with the levels of DON fed in the diets. The DON detected in the frass from larvae fed low and high diets was 15% and 6%, respectively of the DON concentration in the wheat provided as diet. Van Broekhoven *et al.* (2017) also fed a naturally-contaminated diet (4,900 µg/kg DON) to yellow mealworm larvae and detected 14% of the consumed DON in frass. Additionally, we estimated the approximate percentage of DON disappearance after its digestion by the mealworm larvae. By using data from a digestibility trial on yellow mealworm larvae fed an ergot contaminated wheat-based feed (in prep), it was established that greater than 95% of ingested DON was either excreted or biotransform. The DON derivative, 3-ADON, as well as NIV, were also detected in frass from our experiment and, as with DON, they were higher than the levels detected in the larvae or feed. Partial metabolism of DON to 3-ADON, or other metabolites (not analysed or unknown), may explain the appearance of these toxins in the frass. As nivalenol is a common co-contaminant of wheat crops in Canada. We believe that NIV was also present in larvae fed the low and high diets, but at concentrations below the limit of detection. Although the mechanisms underlying DON metabolism and excretion in yellow mealworm larvae are still unclear, enzymatic activity by the mealworm's midgut microbiota and the metabolic and/or enzymatic processes of the mealworm itself may contribute to the toxicokinetics of DON and other mycotoxins. Previous studies on the role of gut microbiota in the detoxification process in yellow mealworm larvae indicated a rapid adaptation of its microbial enzymatic package in response to dietary changes (Genta *et al.*, 2006). Therefore, it is possible that rearing conditions in the present study aided the adaptation of the gut microbiota in the larvae and facilitated the modification of DON into 3-ADON and other metabolites. Genta *et al.* (2006) evaluated the activity of midgut enzymes and gut bacterial enzymes in the yellow mealworms and concluded that an interaction between the two aided the detoxification processes. Hence, microbial and midgut enzymes may lead to the acetylation of DON to 3-ADON and could explain why it was excreted at a higher concentration than that

ingested. Since not all of the DON ingested from the diets was sequestered by larvae or excreted, it is possible that the remaining portion was metabolized into uncommon or unknown metabolites and were thus undetected in our samples. Processes other than acetylation convert DON into uncommon derivatives. For example, de-epoxidation (yielding DOM-1), glycosylation (yielding DON-3G, DON-15G, DON-3-O-glucuronide, DON-15-O-glucuronide), oxidation (yielding 3-oxo-DON), and epimerization (yielding 3-epi-DON) may be pathways used by our larvae to metabolize DON in novel derivatives (Ran *et al.*, 2013). Further studies into the enzymatic processes within mealworms fed DON-contaminated diets could provide insight into DON metabolism in insects and help guarantee the safety of mealworm protein for use in animal feed.

3.4.2 Mealworm performance

The survival rate of yellow mealworm larvae reared on DON-contaminated wheat for 32-34 days was comparable to those reared on the control wheat, indicating that they could tolerate up to 12 000 ug/kg DON. Similar results were reported by Van Broekhoven *et al.* (2017) where the survival rate was 98.3% for yellow mealworm larvae fed a natural DON-infected (4,900 µg/kg) and 99.3% for those fed an artificially-contaminated diet (8,000 µg/kg). Guo *et al*. (2014) observed mortality rates of greater than 20% after challenging the yellow mealworm larvae with different species of *Fusarium* (*F. graminearum* was not evaluated) with the highest mortality in larvae fed kernels infected with *F. bassiana (*59%) and *F. culmorum* (27%). However, DON was not considered the cause of mortality, but rather the presence of other mycotoxins detected in the larvae such as AFB_1, ENN, BEA and ZEA. Van Broekhoven *et al*., (2014) found that yellow mealworm larval survival was not highly affected (99%) when fed diets contaminated with OTA and T-2 toxin. The ability of the yellow mealworm larvae to tolerate high levels of DON may depend not only on the mycotoxin ingested, but also on the fungal species infecting the kernels, as shown by Guo *et al*. (2014). Finally, other factors such as specific metabolic processes, gut microbiota, population density, type of diet and behavior could also influence the survival. Consuming concentrations of DON (up to 12,000 µg/kg) had no direct detrimental effects on yellow mealworm larval growth as weight and ADG were not different than larvae fed the control diet. Larvae fed the control wheat with 15% CP had a higher ECI value (47.3±6.0%), and lower FCR (2.1±0.3) compared to those fed the DON-contaminated diet with 11% CP but there

was no effect on ADG. Van Broekhoven *et al*., (2015) also found the lowest ECI and highest FCR values in the yellow mealworm larvae were associated with low protein/high starch diets versus a high protein/high starch diet. Since survival and larval weight were not affected by the level of DON in the diet, we propose that the differences in FCR, ECI, and PER in mealworms fed our control diets may be related to a poorer assimilation of the nutrients.

3.4.3 Chemical nutritional analysis

The dry matter and ash values in our samples were similar to those of other studies where larvae were fed wheat bran (Ravzanaadii *et al.*, 2012), wheat flour and yeast (Ghaly and Alkoaik, 2009), or organic wastes (Ramos-Elorduy *et al.*, 2002b; Van Broekhoven *et al.*, 2015). In our study, larvae CP values were consistent across treatments (50% protein content) and are similar to those previously reported for the yellow mealworm larvae (Ghaly and Alkoaik, 2009; Ravzanaadii *et al.*, 2012; Van Broekhoven *et al.*, 2015). Larvae CF values in the current study were also similar to those previously reported by Ghaly and Alkoaik. (2009) and Ravzanaadii *et al.* (2012). Only four FA were detected in larvae, in the FDL oleic acid and linoleic acid were the most prevalent followed palmitic acid and by myristic acid. The values of these four FA were similar to those reported by Oonincx *et al.* (2015), Paul *et al.* (2017) and Van Broekhoven *et al.* (2015), except that we did not detect palmitoleic acid, stearic acid or α-linoleic acid. Low amounts of eicosenoic acid and vaccenic acid have been reported in yellow mealworm larvae by Ravzanaadii *et al*. (2012). Fasel *et al.* (2017) studied the fatty acid composition of yellow mealworm larvae after feeding them different ratios and concentrations of ω-3/ω-6 FA. They concluded that the larvae possess plasticity to convert FA into triglycerides easily, and therefore changes in dietary lipids could be reflected in the FA composition of the larvae. Differences in the fatty acid profiles between our larvae and other studies may be a consequence of the different FA contained in our feed. Comparing the FA detected in ODL and FDL, the lower linoleic acid level in ODL could be the result of the higher temperature from the oven-drying method saturating it into palmitic and myristic acid (saturated fatty acids). Myristic acid and palmitic acid were numerically higher in the ODL. Determination of amino acid profiles showed no major numerical differences in FDL samples although minor differences were observed in cysteine between FDL and ODL. It is possible that a partial thermal decomposition of the proteins caused by the heat in the oven-drying method decreased the availability of cysteine in the sample. Our

amino acid (dry matter) results were similar to those of other studies where yellow mealworm larvae were fed organic wastes (Janssen *et al.*, 2017; Ravzanaadii *et al.*, 2012).

Recent research has reported positive effects of chitin on animal health. For instance, chitin acts as an immunomodulator in the European sea bass (*Dicentrarchus labrax*; Henry *et al.*, 2018) and inhibited proliferation of microorganisms such as *Salmonella* and *Escherichia coli* in broilers (Khempaka *et al.*, 2011). For this reason, we wanted to determine whether or not chitin content would change after feeding larvae different levels of DON. Because chitin is structurally similar to cellulose, fiber in our samples was only estimated in the ADF fraction (Finke, 2007; Marono *et al.*, 2015). Our ADF, ADIN and chitin values were lower than those reported by Marono *et al.* (2015), but higher than those found by Finke (2015). The variability between our study and these in terms of fiber suggested that mealworms may assimilate fiber according to the availability and quality in the protein (non-digestible nitrogen) source consumed. In addition, ADF, ADIN and chitin values were slightly higher in ODL, again possibly the result of high temperature used in the oven-drying process. The data obtained after analysing these two drying methodologies suggests minimal differences in the nutrient profile of the larvae. However, of the two methods, the oven-drying method is likely more suitable based on scalability and cost when considering a large-scale mealworm production facility.

3.4.4 Breeding and preference trials

Evaluating survival, developmental time, and production traits in insects can be difficult as multiple factors such as small changes in the environment, availability and quality of feed, and intra-specific interactions can affect such parameters (Weaver and McFarlane, 1990). For example, low population densities have been associated with increased body weights in larvae, pupae and beetles and slow the instar change and pupation rates (Weaver and McFarlane, 1990). This may explain the differences we observed in pupation rate and the additional three days to the end point between the smaller population (n=4,000) reared for the breeding trial compared to the larger population from the feeding trial (n=6,000; Appendix Table 1). There was no effect of diet on larvae and beetle survival or pupal and larval weight. In the second-generation, larval weight was higher than those in first generation (Appendix Table 2). Since the second-generation larvae were exposed to the diets for a longer period of time (approximately 60 days) and were reared in larger containers, it may be that larvae would be heavier as a result of the longer

consumption and lower density. Weaver *et al.* (1989) have described the transfer of microbiota to newly hatched larvae from the consumption of the previous generation's frass, this may have resulted in a better assimilation of the nutrients also increasing weight gain. We observed lower average pupal and beetle weight in the second-generation compared to the first generation (Appendix Table 2). As previously discussed by Weaver and McFarlane (1990) and observed here in our experiment, the increase in density from one generation to the next could have affected the biomass in these two life stages of the yellow mealworm.

We aimed to determine whether or not yellow mealworm larvae preferred diets infected with *F. graminearum.* Unlike Guo *et al.* (2014), we observed no difference in terms of preference or avoidance by yellow mealworm larvae to *F. graminearum*-infected and DON-contaminated diets (Appendix Table 3). Under natural conditions, insects are attracted to rich nutritional material and water sources. Since we supplied our larvae with a constant source of moisture and similar dietary conditions, their nutritional requirements were satisfied and therefore lack of attraction to a specific diet.

In conclusion, yellow mealworm larvae reared on *Fusarium*-infected wheat diets containing up to 12,000 µg/kg DON did not accumulate DON and had minimal to no effect on larvae survivability, nutritional composition and production traits. Further research should investigate the effect of potentially sequestered DON-derivatives that may be produced by the larvae, and that could cause adverse effects in animals. These results support using DON-contaminated wheat in large-scale production of mealworms to produce a sustainable, safe protein source for animal feed.

CHAPTER 4

4. FUTURE DIRECTIONS

In the near future, traditional farming practices will no longer be able to supply enough protein to meet the demands of the world's increasing population. Conventional livestock systems utilize a large land base and consume large amounts of natural resources, such as water, that can negatively impact the environment. For this reason, novel and more sustainable sources of food are being investigated, including alternative, lower-cost feed ingredients for livestock producers. The ancient practice of entomophagy has been garnering the attention of the modern world as insects are easy to grow in low-cost organic residues and may satisfy the nutritional requirements for domestic animals. To date, research in this area aims to produce insect-based ingredients from unusable food by-products. Nevertheless, consuming products of this nature generates a big concern in terms of food safety, as wastes may contain toxic compounds which could negatively impact animal health and productivity.

This thesis research demonstrated that yellow mealworm larvae sequestered low levels of DON after being exposed to wheat contaminated with DON at concentrations as high as 12,000 µg/kg. These results suggest that mealworms grown on DON-contaminated wheat could produce a safe alternative ingredient for animal feed as DON concentrations were far below the regulatory limits established by the CFIA. Regardless of this achievement, further research should evaluate the presence of DON derivative forms (e.g., DON-3G, DON-15G, DOM-1) that may be generated as part of the metabolism of DON by larvae. Additionally, the fate of contaminants co-occurring in salvage wheat such as other mycotoxins, alkaloids, pesticide residues, and heavy metals, should also be analysed when rearing yellow mealworms on salvage wheat. To date, research has evaluated the yellow mealworm tolerance when exposed to diets contaminated with AFB_1, OTA, ZEA and T-2 toxin showing promising results in terms of food safety, and mealworm production and performance (Bosch *et al.*, 2017; Camenzuli *et al.*, 2018; Van Broekhoven *et al.*, 2014). Nevertheless, derivative forms of these other mycotoxins may also be generated. It is a matter of research to investigate their potential accumulation and additive/synergistic effects on health and productivity if included in animal diets.

Future research should also evaluate the effect of this insect-meal in animal trials focusing on health and productivity. So far, most studies have prioritized animal species such as poultry and fish to demonstrate the beneficial effect of the inclusion of mealworm protein from insects reared on organic waste. For instance, the partial inclusion of yellow mealworm meal on broiler diets have improved FCR when compared to conventional soybean meal diets (Bovera *et al.*,

2015), whereas immune system is reportedly enhanced in Atlantic salmon and European sea bass (Gasco *et al.*, 2016; Henry *et al.*, 2018; Karlsen *et al.*, 2017). However, there are no studies demonstrating the effects on animal health and productivity when including protein from insects reared on mycotoxin-contaminated substrates. Currently, our research group is investigating the effect on the inclusion of mealworm-meals from larvae reared on DON-contaminated wheat on broiler chicken performance and health in a feeding trial.

The production of insects using DON-contaminated wheat could be one of the most profitable and sustainable modern livestock systems. Conventional protein ingredients, such as soybean meal and fishmeal, are more sustainable as demand less usage of natural resources. Therefore, rearing insects on salvage wheat would lower the cost of production due to its lack of commercial value and will also offer a more affordable solution for animal protein. As of today, mealworm meal is only approved for use in the fish and poultry industries, which limits its commercial use for other livestock. Further research will be needed to expand the use of mealworm meal in other species. Through our research, we expect to contribute to the legal and societal acceptance of the use of larvae-meal in animal diets, as well as the foundation for a large-scale production of mealworms reared on worthless mycotoxin-contaminated grain in Canada.

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6. APPENDICES

Appendix A. Development traits of yellow mealworm larvae, pupae, and beetles fed four different levels of deoxynivalenol (DON)-contaminated wheat

Parameter	**Treatment**[1]				**SEM**	**P value**
	Control	**Low**	**Medium**	**High**		
Time to endpoint (days)	36.6±1.3	35.2±0.5	35.0±1.9	35.0±2.4	0.742	0.384
Development time (days)	50.6±2.1	52.4±1.8	53.0±1.9	50.4±2.3	0.906	0.148
Survival Larvae (%)	98.4±0.7	99.3±1.1	99.0±1.2	99.1±1.3	0.490	0.609
Pupal survival (%)	$99.6\pm0.6_a$	$98.2\pm0.7_b$	$96.9\pm0.9_b$	$98.4\pm0.9_b$	0.357	0.001
Pupal weight (mg) [2]	125.5±2.5	123.3±5.0	123.7±4.7	127.1±3.3	1.932	0.407
Adult survival (%)	96.6±3.8	96.6±4.1	97.0±2.1	97.8±1.2	1.349	0.907
Adult weight (mg) [2]	103.8±8.1	101.5±4.4	102.3±5.4	105.4±4.3	2.573	0.717

[1]Mean of replicates (n=5) per treatment ± standard deviation

[2]Traits evaluated as average

Values within a row that are followed by a different letter indicate significant differences ($P<0.05$)

Concentration of DON in diets: 200 µg/kg (control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

Appendix B. Average weights (mg) of 200 larvae, 50 pupae, and 100 beetles from the second generation of yellow mealworm fed four different levels of deoxynivalenol (DON)-contaminated wheat

Insect stage	**Treatments**[1]				**SEM**	**P-value**
	Control	**Low**	**Medium**	**High**		
Larval Weight	121.3±4.2	119.7±4.0	116.1±3.8	119.5±5.4	1.965	0.328
Pupal Weight	109.6±3.3	106.0±6.9	105.2±3.0	106.0±7.8	2.530	0.621
Beetle Weight	$88.0\pm4.3_{a}$	$77.8\pm1.5_{b}$	$80.2\pm3.3_{b}$	$76.0\pm3.2_{b}$	1.447	0.001

[1]Mean of replicates (n=5) per treatment ± standard deviation

Values within a row that are followed by a different letter indicates significant differences ($P<0.05$)

Concentration of DON in diets: 200 µg/kg (control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)

Appendix C. Percentage (%) of yellow mealworm larvae that remain in each quadrant 20 minutes after exposure to four different concentrations of DON.

Treatment[1]				SEM	P Value
Control	**Low**	**Medium**	**High**		
23.83±5.06	31.7±8.5	24.5±0.5	20.0±4.8	3.172	0.150

[1]Mean of replicates (n=3) per treatment ± standard deviation

Concentration of DON in diets: 200 µg/kg (control), 2,000 µg/kg (low), 10,000 µg/kg (medium) and 12,000 µg/kg (high)