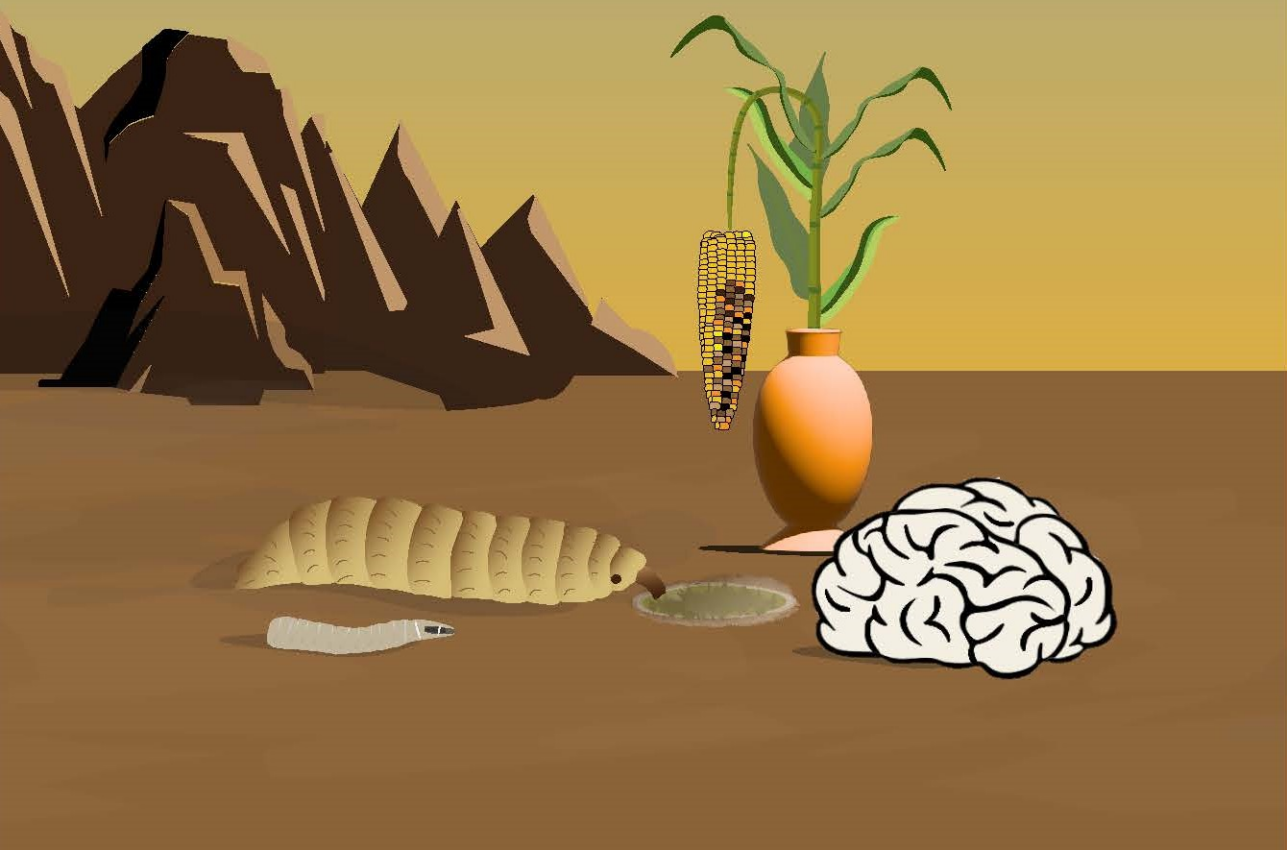
Unravelling mycotoxin biotransformation by the black soldier fly and house fly

Kelly Niermans



Propositions

1. HFL fed on an aflatoxin B_1 spiked diet is safe as food and feed.

   (this thesis)

2. BSFL convert aflatoxin B_1 into the less toxic aflatoxin P_1.

   (this thesis)

3. Land is the most limiting and crucial resource required for the energy transition.
4. The current peer reviewing process is not suitable for multidisciplinary studies.
5. The Dutch school system promotes segregation.
6. One year of social service should be mandatory in the Netherlands.

Propositions belonging to the thesis, entitled

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Kelly Niermans

Wageningen, 12 March 2024

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Kelly Niermans

Thesis
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Table of contents

Glossary

15AcDON	15-acetyldeoxynivalenol
3AcDON	3-acetyldeoxynivalenol
AC	Agroclavine
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFL	Aflatoxicol
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
AKR	Aldo-keto reductase
AME	Alternariol methylether
AOH	Alternariol
BAF	Bioaccumulation factor
BEA	Beauvericin
BSF(L)	Black soldier fly (larvae), *Hermetia illucens* L.
CDC	Centre for Disease Control and Prevention
CIT	Citrinin
Cyb5	Cytochrome b5
CYP450	Cytochrome P450
DAS	Diacetoxyscirpenol
DEG	Differentially Expressed Gene
DM	Dry matter
DOL	Day-old-larvae
DON	Deoxynivalenol
DON3G	DON-3-glucoside
DUOX	Dual oxidase
EAC	East Africa Community
EC	European Commission
EFSA	European Food Safety Authority
EMO	Emodin
ENNA	Enniatin A
$ENNA_1$	Enniatin A_1
ENNB	Enniatin B
$ENNB_1$	Enniatin B_1
ESI	Electrospray ionization
EU	European Union
FA	Fusaric acid
FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FDA	Food and Drug Administration
FP	Fusaproliferin
GO	Gene Ontology
GSH	Glutathione
GST	Glutathione S-transferase

GV	Health-based guidance value
HF(L)	Housefly (larvae), *Musca domestica* L.
HFB_1	Hydroxy-fumonisin B_1
HRMS	High Resolution Mass Spectrometry
HT-2	HT-2 toxin
IARC	International Agency of Research on Cancer
InsectFeed	Research project: Insects as sustainable feed for a circular economy
JHEH	Juvenile hormone epoxide hydrolase
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LFC	Log Fold Change
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
ML	Maximum level
MON	Moniliformin
MOs	Microorganisms
MPA	Mycophenolic acid
MRP	Multidrug resistance protein
nd	Not detected
NIV	Nivalenol
NPA	Nitropropionic acid
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTα	Ochratoxin alpha
PA	Penicillic acid
PACA	Partnership for Aflatoxin Control in Africa
PBO	Piperonyl butoxide
PCA	Principal Component Analyses
PPC	Peanut press cake
ql	Qualifier ions
qn	Quantifier ions
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RASFF	Rapid Alert System for Food and Feed
ROQ	Roquefortine C
RSB	Rwanda Standards Board
SD	Standard error
SDGs	Sustainable Development Goals
SEM	Standard error of the mean
STER	Sterigmatocystin
T-2	T-2 toxin
TeA	Tenuazonic acid
UDP	Uridine diphosphate
UGT	UDP-glycosyltransferases
UN	United Nations
UNEP	United Nations Environment Programme
USA	United States of America
WFSR	Wageningen Food Safety Research

WHO	World Health Organisation
WTO	World Trade Organisation
ww	Wet weight
ZEN	Zearalenone
α-ZEL	α-zearalenol
β-ZEL	β-zearalenol

Chapter 1

General introduction

The global population continues to grow, which is accompanied by challenges. A growing population means an increased demand for food, and associated impacts on biodiversity, land use, food use, climate change, and food security. Furthermore, the world that we live in today is fragile and many challenges are faced by its inhabitants daily. Social (human rights, inequality) and economic injustice, the climate crisis, food shortage and environmental degradation are amongst the biggest problems the world faces.

In the past decades, multiple concepts have been introduced which should help to battle and quantify the severity of these problems. In 2009, the Planetary Boundaries were developed with the aim to guide human development by defining quantitative environmental limits for a set of nine processes that regulate the stability and resilience of the Earths system (Rockström *et al.* 2009a; Rockström *et al.* 2009b). Consequently, exceedance of these boundaries will lead to an increase of the risk on irreversible environmental changes. In 2015, the United Nations (UN) called to action, and 193 countries decided together to protect the planet while promoting prosperity. In order to do this they developed a blueprint for 17 Sustainable Development Goals (SDGs; Figure 1), which are to be collectively achieved by 2030. Most recently, Europe followed with both the European Green Deal (2020; Figure 2) and a proposal for a Sustainable Use Regulation (2022). The latter is a part of the European Union (EU) Farm to Fork strategy focussing on fair, healthy and environmentally friendly food systems and aims to half the use and risk of chemical pesticides by 2030 (EC 2020; EC 2022). The European Green Deal, encompasses a set of policy initiatives proposed by the European Commission (EC) aiming to make the EU climate neutral by 2050 (EC 2019a).

Figure 1. Overview of the 17 United Nations' Sustainable Development Goals (UN 2023)

Figure 2. Overview of the EU Farm to Fork strategy (EC 2023)

SDGs and food security

The UN SDGs should be considered as a universal call to action to protect the planet, end poverty, reduce hunger and inequality, and to ensure peace and prosperity for all world citizens. Next to the topics mentioned before, the 17 SDGs also focus on good health, quality education, clean water and sanitation, renewable energy, economic growth, sustainability, the life below water

and land, and the formation of partnerships needed to make these improvements (Figure 1). Well-being, health, food security and food safety and therefore the availability of safe and nutritious food are considered important to a variety of these SDGs (FAO/WHO/WTO 2019; WHO 2022b). However, the increase in temperature, droughts, rising sea levels and extreme weather events resulting from climate change will impact the global food systems and drive existing and emerging food safety risks (FAO 2020).

It is estimated that by 2050, around 9.7 billion people will inhabit the earth and that, as compared to 2012, the demand for food will increase by 70-80% (UN 2019). At the moment, 49 million people are facing emergency levels of hunger, and up to 828 million people deal with food shortage (WHO 2022a). This situation makes food security (SDG 2, zero hunger) an important cornerstone of the UN 2030 agenda. During the 1974 World Food Summit, food security was defined as "*availability at all times of adequate world food supplies of basic foodstuffs to sustain a steady expansion of food consumption and to offset fluctuations in production and prices*" (UN 1975). However, this definition has been changed and expanded over the years, leading to its most recent definition: "*food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life*" (FAO 2001). The most recent definition clearly shows that food security does not only mean that enough food is available and that everyone has access to it, but that the accessible food also needs to be safe.

Food safety

According to the World Health Organisation (WHO), food-borne illnesses affect 600 million people and cause more than 420,000 premature deaths each year, most of these occurring in low-and middle-income countries (WHO 2016). Foodborne diseases can be both acute and chronic. Contamination can occur during each step in the food chain (cultivation, harvest, processing, storage, transport and consumer handling), and can be a result from pollution of water, air and soil. Public health problems resulting from foodborne diseases heavily impact health-care systems and create a vicious cycle of disease, malnutrition and disability (WHO 2022b). Furthermore, socio-economic growth and local- and global economies are affected due to loss of productivity and trade, which again impacts the global burden of disease as well as food security tremendously (WHO 2016; WHO 2022b). Overall, food safety clearly shows to be very important, and ensuring food safety will play a role in realising SDG 2 (zero hunger), SDG 3 (good health and well-being), SDG 6 (clean water and sanitation) and SDG 8 (decent work and economic growth). Ensuring food safety will not only contribute to the realisation of a variety of SDGs, but is also a requirement for accomplishing the goals set by the EU Farm to Fork strategy (EC 2020) and the WHO global strategy for food safety 2022-2030 (WHO 2022b).

In order to ensure food safety and to protect public health, definitions and legislations are needed (FAO/WHO/WTO 2019; WHO 2022b). For the past 60 years, the joint Food and Agriculture Organization of the United Nations (FAO)/WHO food program (Codex Alimentarius Commission) has developed global standards and guidelines, known as the Codex Alimentarius, which are relevant for food safety. However, even though such standards and guidelines exist, food safety legislations vary between countries (Eskola *et al.* 2020).

Food safety legislation in the EU

In the EU, food- as well as feed safety are regulated by the General Food Law (Regulation (EC) No 178/2002). Article 14 of this law states that food shall not be placed on the EU market when it is either unsafe or deemed to be unsafe, meaning it is injurious to health or unfit for human consumption (EC 2002b). A food can, for example, be considered injurious to health when the health of the person consuming it is affected immediately or on the long term. Additionally, in Article 15, the General Food Law extends its contents to feed safety and states that also feed shall not be placed on the market when deemed unsafe (EC 2002b). Feed is deemed to be unsafe when its consumption can have an adverse effect on human- or animal health or when it makes the food derived from food-producing animals unsafe for human consumption.

EU legislation for contaminants

As by law it is required that food and feed are safe for their consumers, the EC has set limits for a variety of compounds that should protect its consumers and indicate safe levels of consumption for a variety of contaminants. In EU Regulation (EEC) No 315/93 *a contaminant is* defined as *any substance not intentionally added, but present in a food as a result of production, processing, treatment, packaging, transport, storage or as a result of environmental contamination* (EC 1993). In this definition a food contaminant may be biological (*e.g.* bacteria, viruses), chemical (*e.g.* cleaning agents, plant protection products, natural toxins) or physical (*e.g.* pieces of plastic, wood). In this thesis we will only focus the second category: chemical contaminants, and more specifically on the natural toxins. By definition, natural toxins *are poisonous secondary metabolites produced by living organisms, which are typically not harmful to the organisms themselves but can impact on human or animal health when consumed* (Fletcher and Netzel 2020; WHO 2023). Common sources of such toxins are algae, bacteria, fungi and poisonous plants (Fletcher and Netzel 2020).

Food- and or feed may not be placed on the EU market when it contains a contaminant which is unacceptable from a public health viewpoint or when it is present at an unsafe level. In order to ensure this the EU has set maximum levels (MLs), meaning that food or feed with levels of contaminants higher than those specified in their respective legislation may not be sold (EC 2006c). MLs are set at strict levels but should be reasonably achievable by following good agricultural, fishery and manufacturing practices and take into account the risk related to the

consumption of food (no exposure = no risk). Exemptions exist and adjusted rules are applied when a contaminant is considered to be a genotoxic carcinogen. A genotoxic carcinogen is a chemical with the ability to induce carcinogenesis (initiation of cancer formation) by damaging DNA and acts as a mutagen (causes genetic mutation). It is generally considered that for such compounds no safe thresholds exist and that even a single molecule of the compound could cause an adverse effect (EFSA 2005). Next to the MLs, the EU also provides other rules to ensure consumer safety. Health-based guidance values or guidance values in short (GVs), for example, contain a recommendation for the maximum (oral) exposure to a substance that is not expected to result in an appreciable health risk, taking into account current safety data, uncertainties in these data, and the likely duration of consumption (EFSA 2021).

Mycotoxins

Many contaminants could be present in food and feed, however, of major importance are the mycotoxins. Mycotoxins are secondary metabolites which are produced by filamentous fungi (Bennett and Klich 2003). A variety of fungal species are known to produce mycotoxins, and based on their occurrence and preference for certain climatic conditions *e.g.* moisture content and temperature, they might be classified as field (*Fusarium*, *Alternaria*) or storage fungi (*Aspergillus*, *Penicillium*) (Logrieco *et al.* 2003). Currently over 300 different mycotoxins are known to exist of which aflatoxin B_1 (AFB_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), ochratoxin A (OTA) and zearalenone (ZEN) are given most attention, by both regulators and scientists, due to their degree of toxicity and/or prevalence. In 1999 the FAO estimated that around 25% of all globally harvested crops are contaminated by mycotoxins each year (Park *et al.* 1999). However, more recent studies indicated that in 60-80% of the food crops mycotoxins were detected (Eskola *et al.* 2020). Mycotoxins are known to occur in a variety of agricultural commodities (Alshannaq and Yu 2017), and mycotoxin contamination is of global concern as exposure to them can cause detrimental effects on human and animal health (Zain 2011). However, the type of toxicity depends on the type of mycotoxin, the dose and duration of exposure (Bennett and Klich 2003; Chilaka *et al.* 2022).

Furthermore, mycotoxins are the main hazard cited in the EU border rejection notifications system according to the Rapid Alert System for Food and Feed (RASSF) (Alshannaq and Yu 2021; Chilaka *et al.* 2022; Pigłowski 2019). Border rejection due to mycotoxin contamination has financial consequences and directly impacts global trade. On top of that, contamination with mycotoxins is difficult to predict and prevent providing unique challenges to our food safety systems worldwide even when good agricultural-, storage-, and processing practices are implemented. Furthermore, mycotoxins are difficult to eliminate from the food chain due to their stability against heat, physical and chemical treatments (Park 2002).

Mycotoxin contamination is currently considered as one of the most important food/feed safety challenges in the food and feed industry (Moretti *et al.* 2019). Additionally, due to climate change, a shift in the mycotoxin contamination pattern as well as geographical distribution pattern is expected (Zingales *et al.* 2022). Moreover, modelling of different climate change scenarios predicts an expansion of risk zones leading to an increased exposure to these mycotoxins (Battilani *et al.* 2016).

Aflatoxins

Aflatoxins are a group of mycotoxins which are mainly produced by fungal species belonging to the *Aspergillus* genus, in particular *A. flavus* and *A. parasiticus* (Bennett and Klich 2003), which normally occur in soil and other organic materials (Paulussen *et al.* 2017). Aflatoxins are found in a wide variety of foods such as cereals (maize, rice, barley, oats, and sorghum), nuts (peanuts, ground nuts, pistachio, almonds, walnuts), spices, cottonseeds and milk (Alshannaq and Yu 2017; Kumar *et al.* 2016; Park *et al.* 2002; Yakubu and Vyas 2020). Amongst the aflatoxins, AFB_1 is most known due to its genotoxic potential. Other known aflatoxins are aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Bennett and Klich 2003). In general, aflatoxins can cause both acute toxicity and chronic carcinogenicity in humans and animals and are known to have mutagenic, carcinogenic, teratogenic, immunosuppressive and hepatotoxic effects, with the liver as their main target organ (EFSA 2004; EFSA 2006; EFSA 2020a; Zain 2011). More specifically, AFB_1 is considered as a genotoxic carcinogen (EFSA 2020a).

The International Agency of Research on Cancer (IARC) classified all naturally occurring aflatoxins, including AFB_1 as a Group 1 carcinogen (carcinogenic to humans), while AFM_1 is listed in Group 2B meaning it is possibly carcinogenic to humans (IARC 1993).

The human liver cytochrome P450 (CYP450) enzyme system can metabolise AFB_1 into AFB_1-8,9-epoxide (AFBO), which has two isomers *endo*-8,9-epoxide and *exo*-8,9-epoxide. In humans, this metabolic conversion is mainly carried out by CYP1A2 and CYP3A4. AFBO is highly electrophilic and therefore may bind to proteins and nucleic acids. It is *e.g.* known to covalently bind to the N_7 position on guanine resulting in the formation of the adduct AFB_1-N_7-guanine which may lead to carcinogenesis, making AFBO the ultimate carcinogen. AFB_1 can also be metabolized to a number of hydroxylated products. Some of the known AFB_1 metabolites include aflatoxicol (AFL), aflatoxin P_1 (AFP_1), aflatoxin M_1 (AFM_1) and aflatoxin Q_1 (AFQ_1) (EFSA 2020a). In humans, AFQ_1 seems to solely be produced by CYP3A4, whereas AFP_1 is known to be produced by CYP2A3, CYP2A13, and CYP321A1. The DNA-binding potential of AFQ_1 as well as AFP_1 is much lower than that of AFBO, which is why they could be considered detoxification products. AFL on the other hand is found in the cytosolic fractions of liver preparations and is formed by an NADPH reductase, usually in the cytosol. AFM_1 is a hydroxylated AFB_1 metabolite often found in milk

(Henry *et al.* 1999). AFL and AFM_1 retain their DNA-binding activity, so are not considered detoxification products.

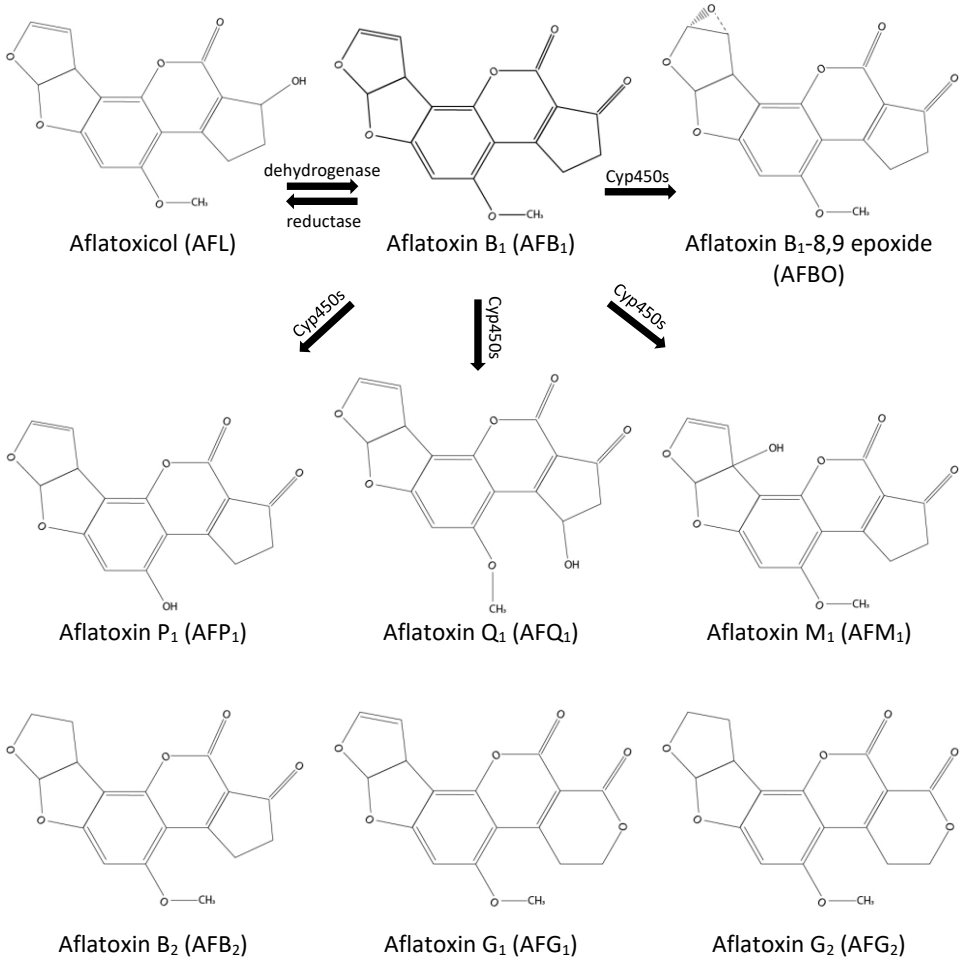

Figure 3: Overview of aflatoxins and the enzymatic degradation pathways documented for AFB_1 metabolites. Figure based on: Bhatnagar *et al.* (2014); Murcia and Diaz (2020a); Rushing and Selim (2019).

Deoxynivalenol

Based on its chemical structure, DON belongs to the trichothecene mycotoxins. Furthermore, DON is considered a *Fusarium* toxin, as it is mainly produced by *F. culmorum* and *F. graminearum*. The most studied metabolites of DON are 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON) and deoxynivalenol-3-β-D-glucoside (DON3G), however also conjugation with a glucuronide or sulphate occurs (Sun *et al.* 2022). DON as well as its metabolites are mainly found in cereals (barley, maize, oats, rye, wheat), and cereal products

like beer, bread, flour and noodles (Alshannaq and Yu 2017; EFSA 2017a). Generally, the acetyl derivatives of DON occur at much lower levels than their parent compound (Chen *et al.* 2022; Yan *et al.* 2020), however, they also are known to cause toxicity *in vitro* and *in vivo* (Pinton *et al.* 2012). Acute effects resulting from high exposure to DON are similar in humans, and some animals, and lead to gastrointestinal symptoms *e.g.* vomiting and feed refusal (Prelusky *et al.* 1994). Chronic effects in animals have been described to be anorexia and weight gain suppression (EFSA 2017a). The IARC has decided that DON is unclassifiable as to its carcinogenicity to humans and is therefore placed in Group 3 (IARC 1993). This classification does not mean that DON has been determined to be non-carcinogenic, but that more information is needed.

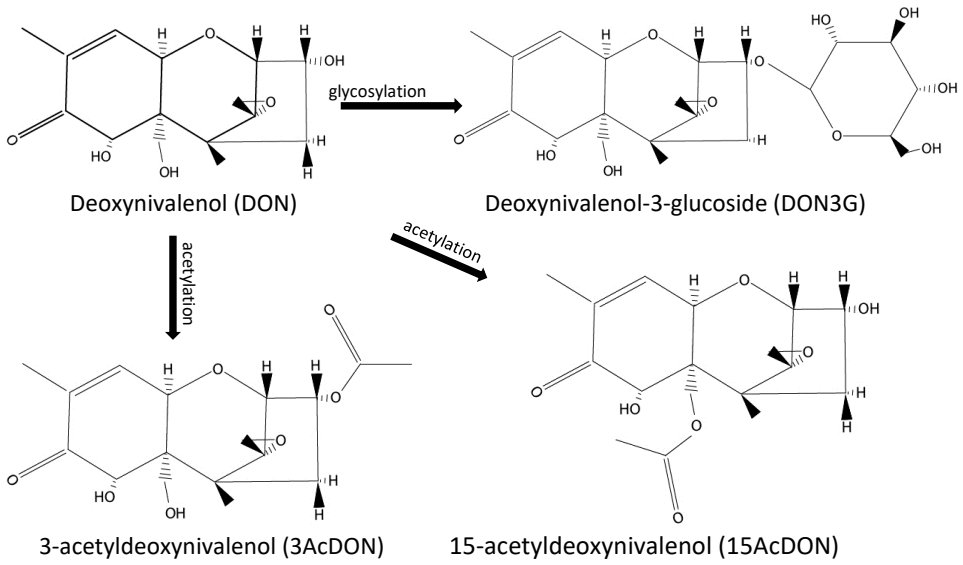

Figure 4: Conjugation of DON into DON3G, 3AcDON and 15AcDON. Figure based on: Fiby *et al.* (2021); Ran *et al.* (2013).

Fumonisins

Fumonisins are mainly produced by fungi from the *Fusarium* genus *e.g. F. verticillioides* and *F. proliferatum*, but can also be produced by some *Aspergillus* strains (EFSA 2018b; Mogensen *et al.* 2010). Around 28 different fumonisins have been characterised, of which FB_1, fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are the most abundant naturally occurring fumonisins (Rheeder *et al.* 2002). FB_1 typically makes up for 70-80% of the fumonisins produced and is therefore the most prevalent fumonisin. Biotransformation of FB_1 into its hydrolysed forms, hydroxy-FB_1 (HFB_1) or p-hydroxy-FB_1, is limited in mammals, whereas extensive biotransformation is observed in pigs and ruminants (EFSA 2018b; Fodor *et al.* 2008; Rice and Ross 1994). FB_1 and its

hydrolysed forms can undergo further biotransformation in via *N*-acylation with fatty acids of various chain lengths (EFSA 2018a; Harrer *et al.* 2015; Seiferlein *et al.* 2007). FB_1 commonly contaminates asparagus, beer, cereals, dried figs, garlic and maize (EFSA 2018b; Heperkan *et al.* 2012; Kawashima *et al.* 2007; Park *et al.* 2002; Seefelder *et al.* 2002). Of the fumonisins, FB_1 is considered most toxic, and is known to trigger multi-organ dysfunction such as pulmonary oedema, hepatotoxicity, nephrotoxicity, immunotoxicity, and disturbance of the intestinal barrier function (Bouhet and Oswald 2007; Chen *et al.* 2021; Loiseau *et al.* 2015). Fumonisins target a variety of organs, but the liver and the kidney can be considered as the main ones affected (Chen *et al.* 2021). IARC classified FB_1 in Group 2B, which means it is considered as possibly carcinogenic to humans (IARC 1993).

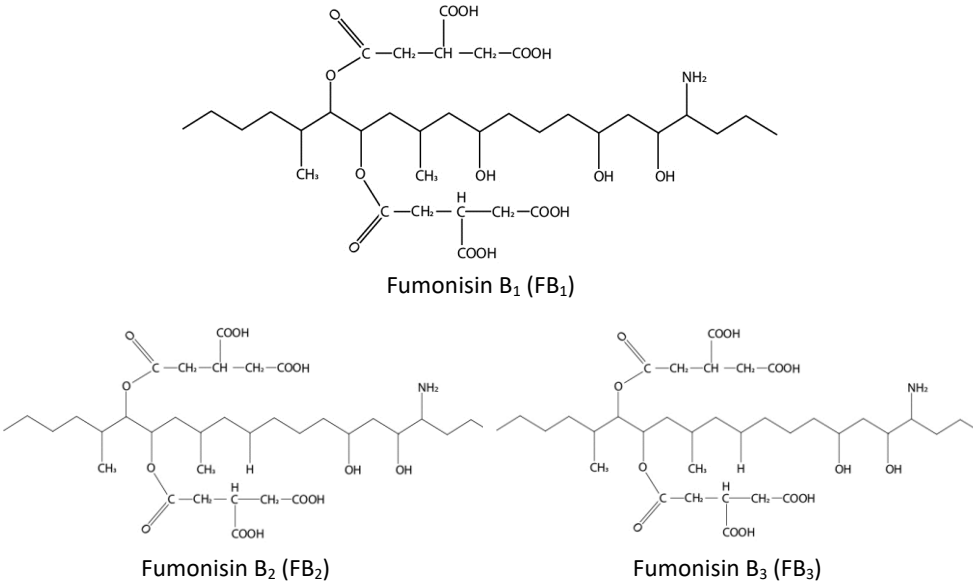

Figure 5: Chemical structures of FB_1, FB_2 and FB_3. Figure based on: EFSA (2018a); Waśkiewicz (2014).

Ochratoxins

The ochratoxins are a group of compounds produced by a variety of fungi of the genus *Aspergillus* and *Penicillium* e.g. *A. ochraceus* and *P. verrucosum*, amongst others (Heussner and Bingle 2015). The most toxic, and therefore most important toxin of the group is ochratoxin A (OTA). Amongst the other ochratoxins are ochratoxin-alpha (OTα), ochratoxin B (OTB) and ochratoxin C (OTC) which are the hydrolysed, non-chlorinated and ethyl ester forms of OTA, respectively (EFSA 2020b). Ochratoxins are often found in agricultural commodities like cereals (barley, corn, oats, rice, rye, wheat), beans, peas, coffee, cacao, spices, grapes, beer and wine

(Alshannaq and Yu 2017; Kawashima *et al.* 2007; Streit *et al.* 2012), but are also present in animal-derived products such as, meat, milk and cheese (Altafini *et al.* 2021; Ganesan *et al.* 2021). OTA is reported to be nephrotoxic (Krogh *et al.* 1979), and at higher doses OTA is also shown to be neurotoxic and carcinogenic (Mantle *et al.* 2005), immunotoxic and teratogenic in both humans and animals (EFSA 2020b). The kidney is the main target organ (EFSA 2020b), and the IARC classified OTA in Group 2B (IARC 1993).

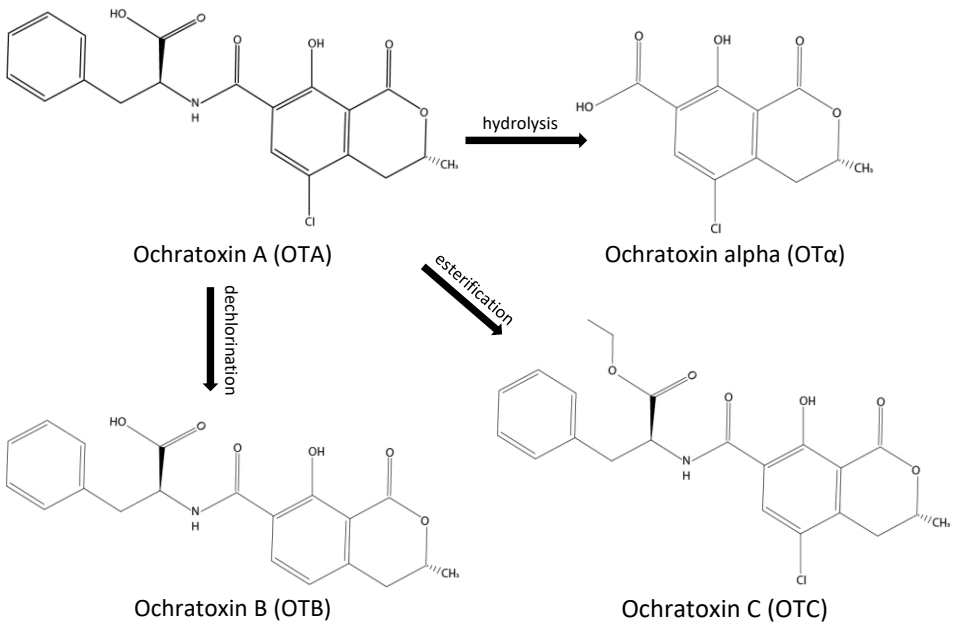

Figure 6: Biotransformation of ochratoxin A into OTα, OTB and OTC. Figure based on: Freire *et al.* (2019); Kőszegi and Poór (2016); Tran *et al.* (2020b).

Zearalenone

Zearalenone (ZEN) is produced by a variety of *Fusarium* species, mainly by *F. culmorum*, *F. graminearum*, *F. verticillioides* and *F. equiseti* (Bennett and Klich 2003). Plants and fungi metabolise ZEN via conjugation with glucose or sulphate (Binder *et al.* 2017). ZEN is mainly found in crops such as barley, maize, rye, sorghum and wheat (Alshannaq and Yu 2017). ZEN toxicity is based on its ability to bind competitively to the oestrogen receptors ERα and ERβ, resulting in adverse effects on the female reproductive system. However, also adverse effects on sex organ weight, spermatogenesis and testosterone synthesis in male animals have been observed (EFSA 2011a). In mammals the main metabolites of ZEN are α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (Miles *et al.* 1996), however, also conjugation with glucuronic acid occurs (Binder *et al.* 2017). α-ZEL shows a higher estrogenic potential than its parent compound. Like DON, ZEN is classified, as a Group 3 carcinogen by IARC (IARC 1993).

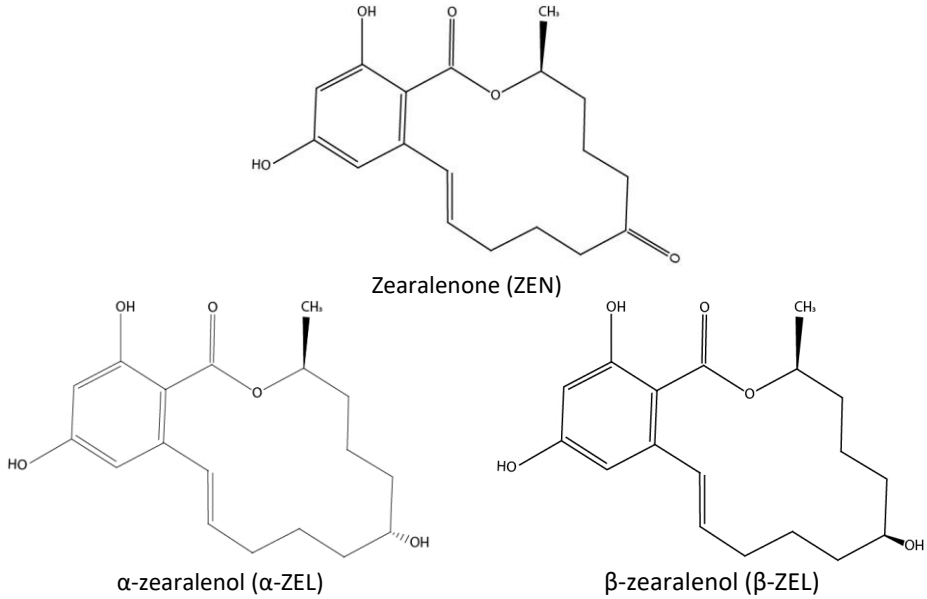

Figure 7: Chemical structures of ZEN, α-ZEL and β-ZEL. Figure based on: Balázs *et al.* (2021); Waśkiewicz (2014).

Emerging mycotoxins

According to the latest definition, emerging mycotoxins are "*mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing*" (Vaclavikova *et al.* 2013). Currently, the *Alternaria* metabolites alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA), the *Aspergillus* metabolites emodin (EMO) and sterigmatocytin (STER), the *Fusarium* metabolites beauvericin (BEA), enniatins (ENNs; A_1, A_2, B_1, B_2), moniliformin (MON), fusaproliferin (FP), fusaric acid (FA), and the *Penicillium* metabolite mycophenolic acid (MPA) are considered to be emerging mycotoxins (Gruber-Dorninger *et al.* 2017; Vaclavikova *et al.* 2013). In general, toxicity data for these mycotoxins are either lacking or the available data is inconclusive. Therefore, more relevant toxicity data are needed in order to draw conclusions regarding their toxicity. On the other hand, it is known that STER shares its biosynthetic pathway with the aflatoxins and has a similar chemical structure as AFB_1. Like AFB_1, STER is considered a genotoxic carcinogen and mainly targets the liver (Díaz Nieto *et al.* 2018; WHO/FAO 2018).

Mycotoxin co-occurrence

The mycotoxins above have been described separately, however, contamination of food and feed with multiple mycotoxins occurs often (Eskola *et al.* 2020; Lee and Ryu 2017). Data from multi-mycotoxin studies even indicate that around 70-100% of the feed samples analysed contained more than one mycotoxin (Streit *et al.* 2012). This so called co-occurrence of mycotoxins happens because some fungal species, in particular *Fusarium* spp. can produce multiple mycotoxins. Furthermore, several toxigenic fungal species can contaminate a food or feed product simultaneously (Lee and Ryu 2017), and humans and animals generally consume a diet composed of a variety of commodities (Streit *et al.* 2012). Most of the current regulatory guidelines and risk assessments are based on toxicity studies performed for individual mycotoxins. However, 'mixture effects' meaning the combined toxicological effects from mycotoxin co-exposure are increasingly studied (Lee and Ryu 2017). Answers to whether co-exposure results in either additive or synergistic effects or whether antagonistic or non-interactive effects might be happening are needed (Lee and Ryu 2017).

Regulatory limits regarding mycotoxins

Amongst all mycotoxins that are regulated for feed in the EU, only for AFB_1, due to its toxicity, an ML exists while for the other mycotoxins a GV is determined. EU MLs for AFB_1 in feed products range from 5 – 20 µg/kg, while the MLs set by the Food and Drug Administration (FDA) of the United Stated of America (USA) vary between 20 - 300 µg/kg (Table 1). Differences between the enforced MLs on mycotoxins in food worldwide are apparent (Table 1).

In reality, the mycotoxin concentration in food- as well as feed commodities often surpasses the legal limits (Gruber-Dorninger *et al.* 2019). In the EU, food commodities which contain mycotoxins in concentrations above their respective MLs or GVs for food are often converted into feed, as the legal limits for feed are higher. At the moment, there is no European law that specifies how to dispose of food products that are affected by mycotoxins. However, EU Regulation does specify that products containing contaminants, including mycotoxins, exceeding the maximum levels should not be placed on the market after mixture with other foodstuffs (EC 2006c). Decontamination and valorisation of mycotoxin-contaminated commodities can be done via various disposal systems *e.g.* landfilling, burying, incineration, anaerobic digestion and composting. Incineration is most effective in terms of disposal as it destroys the mycotoxin molecule, whereas anaerobic digestion and composting allows for the recovery of energy (*i.e*. biogas production) and materials (*i.e.* nutrients for digestate and/or compost) (Cucina and Tacconi 2022). The EC estimated that mycotoxin contamination leads to annual global crop losses of 5 to 10% (EC 2015), and therefore burdens waste management systems (UN 2021; UNEP 2021; WHO 2022b).

Table 1. Range of legal limits of mycotoxins in food and feed in the East Africa Community (EAC), European Union (EU), United States of America (USA) and the Codex Alimentarius in products intended for animal feed and food in µg/kg.

	Feed			**Food**			
Region	EAC[1]	EU[2]	USA[3]	Codex Alimentarius[4]	EAC[3]	EU[2]	USA[3]
AFB_1	5 - 50	5 - 20	20 - 300		5	0.10 - 12	
Total aflatoxins	20 - 300			10 - 15	4 - 20	4 - 15	20
AFM_1				0.5	0.05	0.025 - 0.05	0.5
DON		900 - 8,000	5,000 - 10,000	200 - 2,000		200 - 1,750	1,000
ZEN		100 - 2,000				20 - 400	
OTA		50 - 250		5		2 - 80	
Total fumonisins		5,000 - 60,000	5,000 -100,000	2,000 - 4,000	2,000	200 - 4,000	2,000 - 4,000

[1] Ankwasa *et al.* (2021); EAC (2017); Nishimwe *et al.* (2022); [2] EC (2002a); EC (2006b); EC (2006c); [3] FDA (2000); FDA (2001); FDA (2010); [4] FAO (2003); FAO/WHO (2014).

Socio-economic challenges

Incineration and a strict limitation of mycotoxin-contaminated commodities is not always an option due to *e.g.* a country's socio-economic status (Nji *et al.* 2022; Van Egmond 2002). Cereal crops like maize are highly susceptible for mycotoxin contamination, but are often staple foods in sub-Saharan African countries and are amongst the main cash crops (Nji *et al.* 2022). Good quality, non-contaminated crops are often exported, leaving the poor-quality commodities for home consumption or sale in the informal sector (Meijer *et al.* 2021b; PACA 2020). As mainly the upper middle, lower middle, low-income and the very poor populations make use of the informal sector, strict legal measures may lead to food insecurity and promote a situation where crops complying to the legal limits are available at a premium price. 'Safe' (non-mycotoxin-contaminated) food may become a luxury product and solely affordable for the more wealthy, resulting in inequality and an expected higher disease burden for lower income households (Leroy *et al.* 2015). Furthermore, regulatory entities have mainly focused their attention on the organised formal sector. This made the enforcement of regulatory standards challenging, as the informal sector is responsible for the majority, *e.g.* 90% in Kenya, of transaction of the food supply (Mutegi *et al.* 2018). In order to limit the impact of mycotoxin contamination on food security as well as waste management practices, implementation of a more safe and circular food system is urgently needed.

Insects as a possible solution

It is clear that we are in need of a more sustainable and circular food and feed sector in which unused or unsafe by-products *e.g.* mycotoxin-contaminated commodities can be brought back into the value chain without stimulating inequality nor affecting public health. It is known that insects can recycle agri-food by-products within the food system thereby contributing to circular food systems. Previous studies have shown that a variety of insect larvae can be used to convert different organic side-streams into insect biomass for feed (Gold *et al.* 2020; Gold *et al.* 2018; Van Huis 2013). It is hypothesized that insects could do the same for mycotoxin-contaminated commodities. Furthermore, insect farming can be done at industrial, small-holder farmer and on community scale. In the EU, insects are considered as livestock and therefore the feed they receive needs to comply with the respective MLs and GVs for animal feed (EC 2002a; EC 2006b). Rearing insects on feed containing mycotoxin concentrations above these limits is currently not allowed. Scientific evidence is therefore needed to determine whether this could be a viable and safe solution.

Previously conducted studies already showed that larvae of the black soldier fly (BSFL; *Hermetia illucens* L.; Diptera: Stratiomyidae) are able to recycle a variety of organic streams (Gold *et al.* 2018). Furthermore, others successfully managed to rear BSFL (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Leni *et al.* 2019) and larvae of other relevant insect species, namely the larvae of the

beetles *Tenebrio molitor* (Niermans *et al.* 2019; Van Broekhoven *et al.* 2014; Van Broekhoven *et al.* 2017), and *Alphitobius diaperinus* (Camenzuli *et al.* 2018; Leni *et al.* 2019) on mycotoxin-contaminated substrates. The impact of mycotoxin exposure on larval viability, biomass, and mycotoxin transfer from substrates to larvae remained unresolved. Moreover, a missing fraction in the mycotoxin mass balance in insect rearing systems suggested that mycotoxin biotransformation occurred in insects. The nature and origin of this mycotoxin fraction required elucidation to conclude on possible food safety implications, as well as the larval capacity to biotransform mycotoxins.

This is where the InsectFeed research program came into play. The InsectFeed consortium aimed to generate a high-quality fundamental knowledge on whether the larvae of the BSF and housefly (HF; *Musca domestica* L.; Diptera: Muscidae) can be used as animal feed in poultry production systems (Figure 8). The research conducted within this consortium was divided in seven integrated research topics (work packages; WP) namely; WP1: conceptualization, WP2: insect welfare and ethics of insect production, WP3: insect health as affected by substrate quality, WP4: insect health and immunology, WP5: insect behaviour and welfare, WP6: effects of the use of insects as feed on poultry health and welfare, and WP7: economic robustness of the value chain, including consumer behaviour. This thesis is part of WP3.

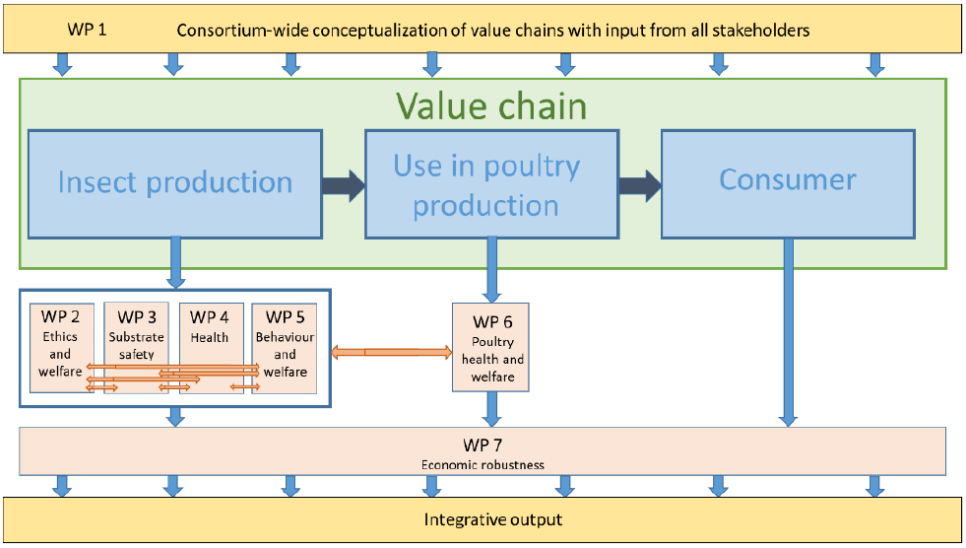

Figure 8: Overview of the work packages (WP) included in the InsectFeed research program.

Objectives and outline of this thesis

The aims of this research topic (WP3) are to (1) determine whether BSFL and HFL survival and biomass are affected after feeding on a mycotoxin-contaminated substrate; (2) whether transfer of the mycotoxins from the rearing substrates into the larvae takes place; (3) whether the insect larvae metabolise the mycotoxins fed and; (4) what metabolic products are formed.

Firstly we aimed to identify knowledge gaps in literature. Therefore, in *Chapter 2* we present a systematic literature review in which we summarized the currently available literature regarding mycotoxin exposure on insects, mycotoxin accumulation and biotransformation. Afterwards and based on the knowledge gaps identified in the literature review we studied the effects of the three mycotoxins AFB_1, DON and ZEN on survival, growth and toxin accumulation in HFL (*Chapter 3*). In this chapter HFL were fed substrates spiked with mycotoxins at concentrations of either one time or 10 times the ML or GVs set for feed materials by the EC. The experiment was performed on laboratory scale and exposure lasted for five days. In *Chapter 4* we focussed on the metabolic fate and metabolic product formation of the mycotoxins AFB_1, FB_1, OTA and ZEN, in both BSFL and HFL and used tracking of an isotopic label in these four compounds in combination with High Resolution Mass Spectrometry (HRMS) analyses to identify the formation of (novel) metabolites.

Afterwards, in *Chapter 5* we examined whether BSFL and HFL were solely responsible for AFB_1 metabolism or whether microorganisms in the feed substrate are also involved. This was done at lab scale with substrates spiked at a concentration of one time the ML for AFB_1, and the preparation of sterile substrates. Lastly in *Chapter 6*, we studied the effects of AFB_1 on metabolic and immunity-related gene expression in BSFL. In order to do this we collected samples at three timepoints during a lab-scale feeding study in which BSFL were fed on an AFB_1-spiked or a control diet and performed RNA-Sequencing to quantify larval gene expression.

The feeding studies in the previous chapters were all performed with mycotoxin-spiked materials, and therefore we studied whether similar results for survival, biomass, mycotoxin transfer and metabolism would be obtained when using peanut material which was naturally contaminated with aflatoxins on laboratory scale (*Chapter 7*). In *Chapter 8* we built further on this and performed a pilot scale study with naturally contaminated maize in Kigali, Rwanda. We mixed mycotoxin-contaminated maize with local agri-food by-products and studied common mycotoxins such as AFB_1, FB_1, DON, OTA, and ZEN, as well as a variety of emerging mycotoxins. Here we furthermore investigated the viability of producing high-quality fertilizer using BSFL from crops contaminated with mycotoxins.

Chapter 2

A systematic literature review on the effects of mycotoxin exposure on insects, and on mycotoxin accumulation and biotransformation

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Abstract

Novel protein sources for animal feed are needed, and the use of insects as feed ingredient is explored. The insect production sector offers opportunities for a circular and sustainable approach to feed production by upgrading waste- or side streams into high-quality proteins. However, potential food or feed safety issues should be studied in advance. Mycotoxins, such as aflatoxin B_1 (AFB_1), are natural contaminants commonly found in agricultural crops and have proven to be detrimental to the agricultural industry, livestock, and human health. This systematic review aims to provide a comprehensive overview of the published evidence on effects of mycotoxin exposure on insect growth and survival, mycotoxin accumulation within the insect body, and metabolization of various mycotoxins by insects. The review includes 54 scientific articles published in the past 55 years, in total covering 32 insect species. The main findings are: (1) insects of the order Coleoptera show lower mortality after exposure to AFB_1 when compared to Lepidoptera and Diptera; (2) effects of mycotoxins on larval growth and survival are less detrimental in later larval stages; (3) accumulation of mycotoxins was low in most insect species; (4) mycotoxins are metabolized within the insect body, the degree of which depends on the particular mycotoxin and insect species; (5) cytochrome P450s (CYP450s) are the main family of enzymes involved in biotransformation of mycotoxins in some insect species. Results of this review support an optimistic outlook for the use of mycotoxin-contaminated waste streams as substrate for insect rearing.

Introduction

With the expected growth of the human population an increase in food and feed production is required and the use of insects as a novel suitable feed source of animal proteins is explored. Opportunities for a circular and sustainable approach to feed production are offered by the insect production sector. Traditionally more than 2000 species of insects are consumed (Jongema 2017), of which most in tropical countries. Beetles (Coleoptera), butterfly and moth larvae (Lepidoptera), ants, bees and wasps (Hymenoptera) are consumed most commonly, followed by crickets, grasshoppers and locusts (Orthoptera) (Van Huis *et al.* 2013). However, in Europe the consumption of insects is still considered novel. Besides increasing the use of insects as food, their use in the feed sector provides interesting opportunities. By using waste- or side streams as substrates for insect rearing low-quality streams can be upgraded into high-quality proteins. But possible issues regarding food or feed safety should be studied beforehand. Serval classes of contaminants could possibly be present in waste- or side streams of interest. When using these waste- or side streams as a substrate to rear insects as food and/or feed it is important to know whether these insects accumulate the possibly present contaminants in their bodies and therefore become a source of contaminants themselves. Waste- or side streams can originate from a variety of sources. This wide variety of available waste- or side streams could also result in contamination by heavy metals, veterinarian drugs and hormones, pesticides,

dioxins, dioxin-like polychlorinated biphenyls and polyaromatic hydrocarbons, among others. Additionally, an example of a group of contaminants commonly found in nature and in agriculture are mycotoxins (Van der Fels-Klerx *et al.* 2018). Mycotoxins are a chemically diverse group of low-molecular weight secondary metabolites produced by fungi, mainly *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp.. Mycotoxins can cause a variety of adverse effects on human and animal health (Hussein and Brasel 2001) and are commonly found in seeds, nuts and ears of crops (Agriopoulou *et al.* 2020). Therefore waste- or side streams consisting of agricultural materials, for example restaurant waste or brewery spent grains, could be contaminated with these mycotoxins and be fed to insects when used as substrates for insect rearing. As mycotoxins are detrimental to both human and animal health, the European Union (EU) has set maximum levels (ML) for the presence of certain mycotoxins in food and food commodities in Commission Regulation (EC) No 1881/2006 (EC 2006c). MLs and advised guidance values (GVs) for feed materials and complementary and complete feeding stuffs in Directive 2002/32/EC and Commission Recommendation 2006/576/EC (EC 2002a; EC 2006b). Table 1 gives an overview of the range of MLs and advised GVs set for feed and food materials.

For a detailed overview of which ML or GV is set for a specific product intended as animal feed or as food we direct the reader to the legal documents themselves. Data on the occurrence of mycotoxins in raw materials used for animal feed production (maize, wheat, barley and soybean) from 100 countries, collected in the past 10 years showed that mycotoxin concentrations mostly complied with the ML or GV set for animal feed in the EU (Gruber-Dorninger *et al.* 2019).

Table 1. Overview of the range of maximum levels (MLs) and advised guidance values (GVs) set for products intended for animal and human consumption

Mycotoxin	MLs in µg/kg	GVs in µg/kg
All feed materials and complementary and complete feeding stuffs		
AFB_1	5 - 20 [1]	
DON		900 - 12,000 [2]
Sum of FB_1 and FB_2		5,000 - 60,000 [2]
OTA		50 - 250 [2]
ZEN		100 - 3,000 [2]
Foodstuffs		
Sum of AFB_1, B_2, G_1 and G_2	4 - 15 [3]	
DON	200 - 1,750 [3]	
Sum of FB_1 and FB_2	200 - 2,000 [3]	
OTA	0.50 - 10 [3]	
Patulin	10 - 50 [3]	
ZEN	20 - 200 [3]	

[1]Directive 2002/32/EC, [2]Commission Recommendation 2006/576/EC, [3]Commission Regulation (EC) No 1881/2006. Aflatoxin B_1 (AFB_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), ochratoxin A (OTA), zearalenone (ZEN), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2)

However, the percentage of samples exceeding the ML or GVs varied between 2.4-7.4% for AFB_1, 4.8-13.0% for zearalenone (ZEN), 4.3-21.5% for deoxynivalenol (DON), 0.2-0.9% for ochratoxin A (OTA) and 0.0-3.3% for fumonisin B_1 (FB_1) + fumonisin B_2 (FB_2). This study showed that mycotoxin presence in feed and related commodities greatly varies from year-to-year and varies between regions of Europe, being highly affected by environmental conditions and agricultural practices (Gruber-Dorninger *et al.* 2019). Climate change and bad storage conditions may result in elevated levels of mycotoxins in plants and crops (*e.g*. maize) on which insects feed (Gruber-Dorninger *et al.* 2019; Medina *et al.* 2017). Some insect species have a degree of tolerance to particular mycotoxins and - in general - insects seem to be able to grow on plant-derived (waste) streams which contain mycotoxins (Bosch *et al.* 2017; Leni *et al.* 2019; Niu *et al.* 2009; Ochoa Sanabria *et al.* 2019). When comparing the initially present mycotoxin concentration with the concentration found in the residual feed material and the larvae, a portion of the ingested mycotoxins could not be recovered (Leni *et al.* 2019; Schrögel and Wätjen 2019) pointing at a need to further investigate the fate of mycotoxins. These unrecovered mycotoxin fractions could indicate the formation of mycotoxin metabolites (Berenbaum *et al.* 2021), or adducts (with protein or DNA), of which some could be unknown. Possible metabolites and modified forms of mycotoxins formed after ingestion of mycotoxins by insects may still be toxic (*e.g.* formation of a more toxic metabolite) to animals or humans (EFSA 2016), indicating the necessity for more information on the metabolism of mycotoxins by insects especially if intended for use as feed and food.

Over the past years the topic of insects as food and feed has received increasing attention and been elaborately discussed by Arnold van Huis (Van Huis 2016; Van Huis 2020; Van Huis *et al.* 2013). Also, the topics of sustainability in insect rearing (Van Huis and Oonincx 2017), consumer acceptance (Kauppi *et al.* 2019), profitability (Niyonsaba *et al.* 2021) and the circular business model perspective (Madau *et al.* 2020) have been extensively discussed in recent reviews. To date, the available data on possible accumulation of mycotoxins in insects and the possible metabolization of these toxins by insects, as well as the possible data gaps, are fragmented across disciplines and no clear overview is available. Therefore, the aim of this study was to obtain a comprehensive overview of the available information on the effects of mycotoxin exposure on growth and survival of insects, possible accumulation of mycotoxins in insects, and the possible bio-transformation of mycotoxins by insects. To this end, a systematic review was done covering all insect species, but with a particular focus on species used for feed and food. This comparative approach may allow extrapolation to a wider range of insect species that may be used for feed and food production in the future.

Methods

Three different bibliographic databases (*i.e.* PubMed, CAB Abstracts and Scopus) were used to retrieve peer-reviewed studies published in the English language from 1950 up to and including

2020. Search strings were defined beforehand and were divided into two parts. The general keywords added in both sets of search strings were: *larva(e), larval, insect(a), insects, mycotoxin(s), deoxynivalenol*, enniatin*, beauvericin*, nivalenol*, aflatoxin*, zearalenone*, fumonisin** and *ochratoxin**. The first search focused on survival and development of insects and included the following additional keywords: *life cycle stage(s), life*cycle, life stages, biomass, reprod* fitness*, grow*, develop*, mortality, weigh*, pupat** and *surviv**. The second search focused on accumulation and transformation of mycotoxins and contained the following additional key-words: *metabolism*, convert, conversion, breakdown, degrad*, accumulat*, conjugat*, absorb*, excret*, distribut* and adme*.* For all search strings used it was ensured that respective plural forms as well as related words (synonyms) were covered. The collected articles were stored in an EndNote library after which duplicates were removed. Then, the articles were screened for their relevance by using *a priori* determined exclusion criteria. Exclusion criteria used included: non-English articles, non-research articles, review articles, no full text available (via the WUR library), not focusing on insects (*i.e.* class Insecta), studies in insect cell-lines, not focusing on mycotoxins, focusing on pest management. The snowballing technique was used to identify other relevant studies from reference lists of the articles found.

Data reported in the relevant articles were extracted and synthesized to provide an overview of effects of mycotoxins on insect mortality and growth, on accumulation of mycotoxins, and on conversion of mycotoxins. Throughout the remaining part of this review, metabolism is defined as the process of biotransformation facilitated by enzymes to create polar compounds which are more easily excretable. The words metabolism, conversion, and biotransformation of mycotoxins will be used interchangeably to refer to metabolic processes which convert mycotoxins within insects into their metabolites or modified forms.

Results

Literature search

The initial literature search yielded 1,282 articles (Figure 1). Following elimination of duplicates and screening of title, keywords and abstract using the exclusion criteria, 148 potentially relevant papers were selected. An additional 24 potentially relevant papers were added in this step via the snowballing technique. After examination of the full texts, 52 papers were considered relevant. Additionally, two relevant papers which were published during the time this review was written were added. All study details and data from the final set of 54 research articles are presented in Table S1, including: insect species, substrate used, exposure time of the insects, analytical method used, and mycotoxins (metabolites) analysed. Although all insect species were included in the review, most of the retrieved studies focused on species from the insect orders Diptera, Coleoptera, and Lepidoptera. The order Diptera includes fly species that can feed on a variety of organic residues and usually have a short life cycle. The Coleoptera are beetles and

include known agricultural pests that can break down animal and plant debris. The Lepidoptera order includes butterflies and moths, the plant-feeding larvae of which are called caterpillars that can be detrimental to agriculture. The majority of currently considered edible insect species belong to the orders Coleoptera and Lepidoptera (Jongema 2017).

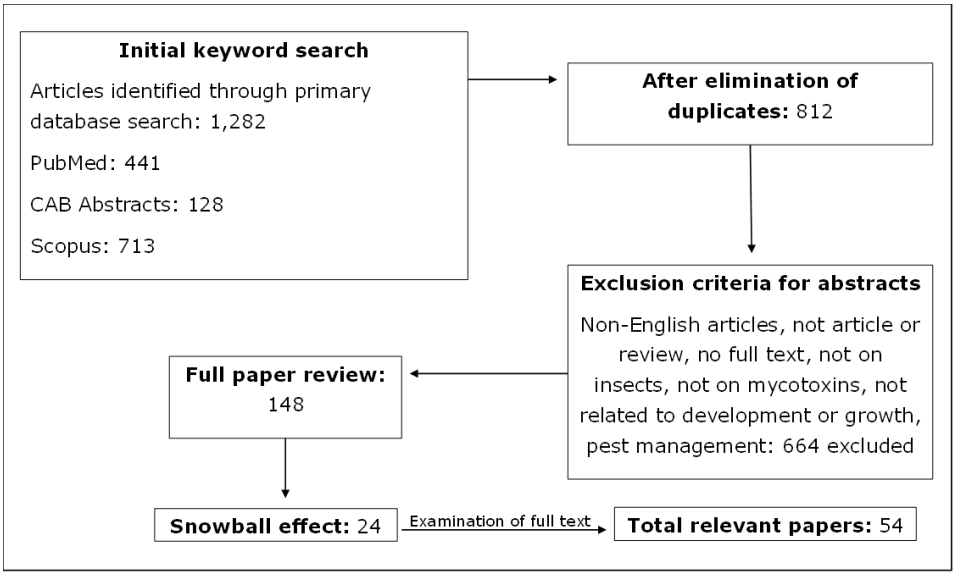

Figure 1. Overview of steps with number of articles of the systematic review process

Development and growth

Diptera

Developmental effects due to exposure of mycotoxins were observed mostly for larvae of *Hermetia illucens* L. and *Drosophila melanogaster* Meigen within the order of Diptera. Larval weight gain of *H. illucens* consuming a substrate contaminated with 4.600 µg/kg DON, 260 µg/kg OTA and 88 µg/kg AFB_1, 17 µg/kg AFB_2, 46 µg/kg aflatoxin G_2 (AFG_2) and 860 µg/kg ZEN was not significantly different as compared to the control treatment (Purschke *et al.* 2017). A significantly longer development time was observed in *Drosophila simulans* Sturtevant female larvae when exposed to 0,05 µg/kg OTA as compared to the control (Cao *et al.* 2019). Furthermore, as the concentration of AFB_1 increased, more malformed adult (wings, leg and thorax) individuals of *D. melanogaster* were observed. Exposure to 800 µg/kg AFB_1 resulted in 11% malformation as opposed to 7% for larvae exposed to 200 µg/kg AFB_1 (Şişman 2006). Furthermore, feeding *D. melanogaster* (strain Oregon R) with 10,000 µg/kg AFB_1 resulted in a doubling of larval and pupal development time (Kirk *et al.* 1971). Additionally, it has been shown that effects caused by mycotoxin exposure of *D. melanogaster* on growth can vary between both strains and larval stages. For instance, when 1st, 2nd and 3rd instar larvae of strain A-11 of *D. melanogaster* were

reared on 440 µg/kg AFB_1 no significant growth effects were observed, however, 2nd instar larvae of strain A-9 raised on 440 µg/kg AFB_1 developed into significantly smaller adults than their controls (Chinnici *et al.* 1979). In addition, *D. melanogaster* larvae fed with 200 µg/kg AFB_1 led to a significantly smaller body length for larvae of the Florida-9 strain (Gunst *et al.* 1982).

Coleoptera

Growth effects caused by mycotoxin exposure were also observed in the order of Coleoptera. For example, developmental time of one-day-old *Ahasverus advena* Waltl larvae was significantly longer when exposed to 2,000,000 µg/kg AFB_1 (Zhao *et al.* 2018). Lifetime fecundity of *Tribolium confusum* Jacq. adults fed with 100,000 µg/kg T-2 for 120 days was not affected, however, this exposure resulted in a much higher egg production in the first 60 days, followed by a lower egg count in the last 60 days (Wright *et al.* 1976). Nonetheless, most available studies focused on the effect of mycotoxin exposure on larval weight. *Alphitobius diaperinus* Panzer larvae fed with 1,300 µg/kg OTA had a significantly lower weight when compared to larvae fed with 1,700 µg/kg OTA and the control (Camenzuli *et al.* 2018). Also, a lower weight was observed for *Zophobas atratus* Fabr. larvae fed on 500 µg/kg T-2 as compared to their control (Van Broekhoven *et al.* 2014). Most studies used *Tenebrio molitor* L. larvae and showed, for example, that exposure to 450,000 µg/kg FB_1 resulted in a significantly lower weight in *T. molitor* larvae after 28 days (Abado-Becognee *et al.* 1998). Furthermore, *T. molitor* larvae gained significantly less weight when exposed to wheat bran contaminated with 8,000 µg/kg DON for two weeks and this effect became more pronounced at increasing concentrations (Janković-Tomanić *et al.* 2019). Contrarily, *T. molitor* larvae exposed to either 500 µg/kg OTA, 500 µg/kg T-2 (Van Broekhoven *et al.* 2014) or 204 µg/kg AFB_1 (Bosch *et al.* 2017) gained more weight than their respective controls, whereas no significant difference in weight gain of *T. molitor* larvae exposed to 415 µg/kg AFB_1 was observed (Bosch *et al.* 2017). Additionally, exposure to flour that was naturally contaminated with 2,854 µg/kg DON and 602.3 µg/kg ZEN resulted in a significantly increased larval weight (Niermans *et al.* 2019). *T. molitor* larvae fed with an artificial diet containing approx. 250 µg/kg T-2 / HT-2 toxins gained 44% more weight than the control group fed a natural diet. Additionally, larval weight gain was significantly higher when fed with an artificially contaminated diet than when fed with naturally contaminated diets (Piacenza *et al.* 2020). In general, most studies focused on the effect of mycotoxin exposure on weight gain in *T. molitor* larvae.

Lepidoptera

Growth effects caused by mycotoxin exposure on Lepidoptera were reported in several studies. Firstly, 1st instar *Amyelois transitella* Walker larvae fed with 50,000 µg/kg AFB_1 showed significantly lower pupation rates. Exposure of 5th instars to the same concentration did not cause a significant decrease in pupation rate. No developmental effects were observed in 1st

instar *Am. transitella* larvae after exposure of up to 5,000 µg/kg OTA, whereas 1st instar *Helicoverpa zea* Boddie larval development was significantly inhibited by this concentration of OTA (Niu *et al.* 2009). Exposure of *Spodoptera frugiperda* Smith and *Hel. zea* larvae to 25,000 µg/kg verrucolgen, roseotoxin B or penitrem A for 7 days resulted in a significantly lower weight gain for both species. Additionally, exposure of *Hel. zea* larvae to 250 µg/kg penitrem A also resulted in a significantly lower weight, this was not observed when the larvae were exposed to the same concentration of roseotoxin B (Dowd *et al.* 1988). Exposure to 25,000 µg/kg DON caused a significant growth retardation in *Hel. zea* larvae but not in *S. frugiperda* larvae. In contrast, exposure to 25,000 µg/kg T-2 resulted in a significantly lower weight of *S. frugiperda* larvae only. However, 25,000 µg/kg diacetoxyscripeol caused a significant growth retardation in both species (Dowd 1990), whereas exposure to 250,000 µg/kg griseofulvin caused significant weight loss in both *Hel. zea* and *S. frugiperda* larvae (Dowd 1993). Similarly, no significant difference in weight of *Hel. zea* larvae was observed after exposure to 25,000 and 250,000 µg/kg fusaric acid for seven days (Dowd 1989). Paterson *et al*. (1990) reared *S. frugiperda* larvae on substrates containing either brevianamide A, brevianamide D, or OTA, each at 10,000 µg/kg, and observed a significant larval weight loss after three days of exposure to each of the three mycotoxins. Exposure to brevianamide D reduced larval weight more than brevianamide A (Paterson *et al.* 1990). In neonatal *Spodoptera exigua* Hübner larvae reared for seven days on a semi-synthetic diet containing 15,000-90,000 µg/kg destruxin B resulted in significant decrease in growth with increasing concentration (Rizwan-Ul-Haq *et al.* 2009). Destruxins are produced by the documented insect pathogenic fungus *Metarhizium spp.*, but they are not common contaminants of food or feed.

AFB_1 and its metabolites caused effects on fecundity and hatchability in *Spodoptera littoralis* Boisduval larvae. Exposure to 2,500 µg/kg AFB_1 or 4,000 µg/kg AFG_1 caused significant retardation in the development of both larvae and pupae, and significantly reduced the percentage of hatchability. Exposure to either 2,000 µg/kg AFB_1, 3,000 µg/kg AFG_1 or 4,000 µg/kg AFB_2 caused a significant reduction in the numbers of eggs laid (Sadek 1996). Tolerance of *Trichoplusia ni* Hübner larvae to AFB_1 seems to increase with age. As an example, exposure of newly hatched larvae to a semi-synthetic wheat germ-based diet containing 200 µg/kg AFB_1 resulted in a significant inhibition of larval growth after ten days, while no negative effects on growth and development were observed in five-day-old-larvae exposed to the same concentration for three days. Additionally, exposure of seven-day-old larvae to 3,000 µg/kg AFB_1 significantly reduced pupation, while exposure of 10-day-old larvae to the same concentration did not affect pupation (Zeng *et al.* 2013). 10-day-old *Corcyra cephalonica* larvae needed to be exposed to at least 1,000,000 µg AFB_1/kg for twelve days to observe a significantly reduced growth (Hegde *et al.* 1967). In *Bombyx mori* L. larvae, oral administration of up to 16,000 µg/kg bassianolide decreased body weight with an increasing dose already after two days. Larvae exposed to 4,000 µg/kg bassianolide weighed half of the control group, however, statistical

significance was not calculated in this study (Kanaoka *et al.* 1978). Bassianolide is produced by the well-known insect pathogenic fungus *Beauveria bassiana*, but is not a common contaminant of food or feed. *Choristoneura fumiferana* Clemens larvae grown on *Picea glauca* branches infected with rugulosin-producing endocytes showed that the *Ch. fumiferana* larvae grown on infected trees containing 850 µg/kg rugulosin (geometric mean) were significantly smaller than the ones grown on uninfected trees (Miller *et al.* 2008). Sumarah *et al*. (2008) performed a similar experiment and observed a significant reduction of growth of *Ch. fumiferana* and *Lambdina fiscellaria* Guenée larvae after exposure to 13,650 and 27,125 µg/kg rugulosin, respectively. A significant reduction of the head capsule was observed in *Ch. fumiferana* larvae fed with 54,250 µg/kg dietary rugulosin and in *L. fiscellaria* larvae fed with 81,375 µg/kg dietary rugulosin. However, larval weight of *Zeiraphera canadensis* Mutuura & Freeman did not significantly differ when exposed to up to 81,375 µg/kg rugulosin, which was the highest concentration tested (Sumarah *et al.* 2008).

Other orders

Periplaneta americana L. (Blattodea) fed sucrose contaminated with 12,000 µg/kg AFB_1 had a higher body weight (approximately 7%) as compared to the control (Llewellyn *et al.* 1976). However, *Oncopeltus fasciatus* Dallas (Hemiptera) were observed to have a significantly lower body length after feeding on 5,000 µg/kg AFB_1 at 20°C, whereas no effect on body length was observed when exposed to the same concentration at 25°C (Llewellyn *et al.* 1988).

Mortality

Diptera

Hermetia illucens and *D. melanogaster* differed in tolerance to AFB_1 exposure (Figure 2a). When exposed to 1–500 µg AFB_1/kg, < 30% mortality was observed in *H. illucens* (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Meijer *et al.* 2019), while exposure to 440 µg AFB_1/kg caused 100% mortality in *D. melanogaster* strain A-9, but only 9% mortality in *D. melanogaster* strain A-11 (Chinnici *et al.* 1979). Exposure to 200 µg AFB_1/kg resulted in mortality of 66% and 9% in the *D. melanogaster* strains Florida 9 and Lausanne-S respectively (Gunst *et al.* 1982). Interestingly, when intermated lines of *D. melanogaster* (strains Oregon-R and Lausanne-S) were exposed to AFB_1 for multiple generations a significantly enhanced resistance as compared to the control line was observed (Melone and Chinnici 1986). While most studies focused on AFB_1, exposure of *H. illucens* larvae to DON in concentrations ranging from 630-3,580 µg/kg resulted in mortality varying between 7-10% (Gulsunoglu *et al.* 2019). On the contrary, no significant difference in mortality was observed when exposing *H. illucens* larvae to a substrate that contained a mixture of DON (4,600 µg/kg), 88 µg AFB_1/kg, 17 µg AFB_2/kg, 46 µg AFG_2/kg, 260 µg OTA/kg and 860 µg ZEN/kg (Purschke *et al.* 2017). Exposure of *H. illucens* larvae to spiked concentrations of AFB_1 (390 µg/kg), DON (112,000 µg/kg), ZEN (13,000 µg/kg) and OTA (1,700 µg/kg) caused ≤6%

mortality (Camenzuli *et al.* 2018). Additionally, 2% mortality was observed in 4th instar *Aedes aegypti* L. larvae when exposed to 3,000 µg AFB_1/kg for 5 days (Matsumura and Knight 1967).

Coleoptera

In the order of Coleoptera, studies were performed on *T. molitor, Ah. advena, A. diaperinus, Z. atratus* and *Tri. confusum*. Similar to the order of Diptera, differences in mycotoxin susceptibility were identified between Coleoptera species (Figure 2b). Mortality in one-day-old *Ah. advena* larvae caused by AFB_1 seems to be dose-dependent and ranged from 29% when exposed to 500,000 µg AFB_1/kg up to 99% when exposed to 8,000,000 µg AFB_1/kg (Zhao *et al.* 2018). *T. molitor* larvae have overall proven to be quite tolerant to various mycotoxins and as shown in Figure 2b - exposure to 415 µg AFB_1/kg resulted in only 5% mortality (Bosch *et al.* 2017). Additionally, no significant effects on mortality were observed when *T. molitor* larvae were fed with contaminated diets containing 200-12,000 µg DON/kg, and exposure to substrates infested with 12,000 µg DON/kg led to 2% mortality (Ochoa Sanabria *et al.* 2019). A naturally contaminated diet containing 4,900 µg DON/kg and another diet spiked with 8,000 µg DON/kg (Van Broekhoven *et al.* 2017) also resulted in 2% mortality. In addition, *T. molitor* larvae fed with wheat containing 568-4,588 µg DON/kg and 589-2,283 µg ZEN/kg did not result in significant mortality (Niermans *et al.* 2019). 7% mortality was observed in *T. molitor* larvae when fed with 128,000 µg T-2/kg (Davis and Schiefer 1982), but no mortality was observed upon exposure to 450,000 µg FB_1/kg (Abado-Becognee *et al.* 1998). However, 100% mortality was observed in *T. molitor* larvae after exposure to 102,280 µg beauvericin/kg (*Beauveria bassiana* strain B13/I11) (Cito *et al.* 2016). It should be noted that beauvericin is produced by the well-known insect pathogenic fungus *Beauveria bassiana*, and is a common contaminant of grains (EFSA 2014). Larvae fed with either an artificially contaminated diet or a naturally contaminated diet containing approximately 100 and 250 µg/kg total T-2 / HT-2 for four weeks resulted in an average mortality of 11% in *T. molitor* larvae considering all diets. Interestingly, when fed the artificially contaminated diet, 16% higher mortality was observed as compared to the naturally contaminated diet, and this effect seemed to be independent of the concentration (Piacenza *et al.* 2020).

Camenzuli *et al*. (2018) exposed *A. diaperinus* larvae to 17 different treatments, including single spiked concentrations of AFB_1 (8-390 µg/kg), DON (3,900-112,000 µg/kg), ZEN (280-13,000 µg/kg), OTA (170-1,700 µg/kg) and combined spiked mycotoxin concentrations of up to 100,000 µg/kg and found no significantly different mortality in these treatment groups as compared to the control group (Camenzuli *et al.* 2018). In another study, exposure to 500 µg/kg ZEN, OTA, and T-2 led to 10% mortality in *T. molitor* and *Z. atratus* larvae, and 20% mortality for larvae of *A. diaperinus* (Van Broekhoven *et al.* 2014). A similar high mortality of 15% was observed in *Tri. confusum* larvae fed on 100,000 µg T-2/kg (Wright *et al.* 1976).

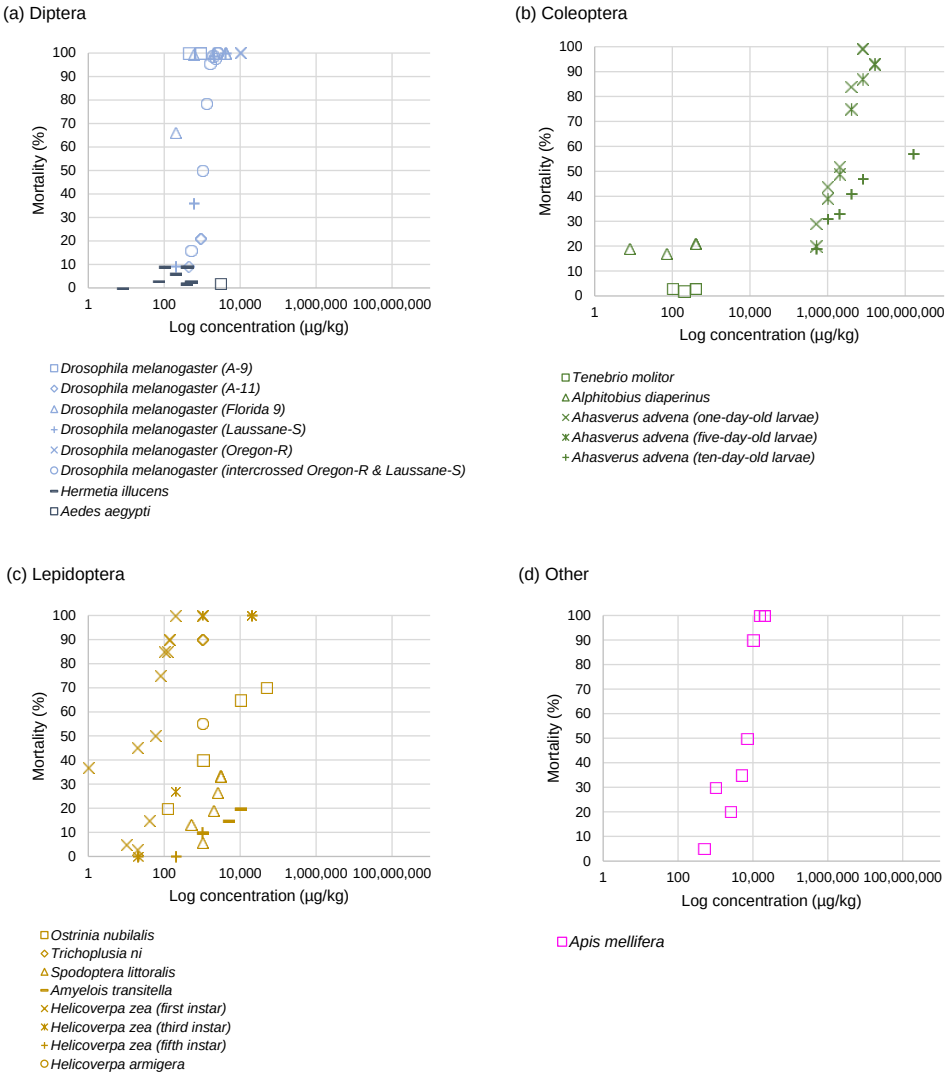

Figure 2. Mortality (%) caused by exposure to different doses of AFB_1 (log scale) for 14 insect species belonging to four orders.

Based on the studies of a: Camenzuli *et al*. (2018), Chinnici *et al*. (1979), Gunst *et al*. (1982), Kirk *et al*. (1971), Matsumura and Knight (1967), Meijer *et al*. (2019), Melone and Chinnici (1986); b: Bosch *et al*. (2017), Camenzuli *et al*. (2018), Zhao *et al*. (2018); c: Mencarelli *et al*. (2013), Niu *et al*. (2009), Sadek (1996), Zeng *et al*. (2006), Zeng *et al*. 2013; d: Niu *et al*. (2011). An overview of the data used is available in Table S2

Lepidoptera

Most studies on the effects of mycotoxin exposure on insects are performed for species in the order Lepidoptera. Similar to the orders previously described, different degrees of susceptibility in the species of this order were observed (Figure 2c) (Niu *et al.* 2009; Zeng *et al.* 2006). First instars of *Hel. zea* exposed to 1,000 µg AFB_1/kg, showed 100% mortality after 15 days, while also 100% mortality was observed in 3rd instars exposed to 20,000 µg AFB_1/kg for 21 days (Zeng *et al.* 2006). Additionally, exposure to 1,000 µg AFB_1/kg resulted in a significant increase in mortality (40-55%) after 6-9 days in *Hel. armigera* larvae (Elzaki *et al.* 2019), (35-55%) mortality after 8 days in *Hel. zea* larvae (Zeng *et al.* 2009), and 90% mortality after 10 days in *Tr. ni* larvae (Zeng *et al.* 2013). *S. littoralis* larvae fed with a diet containing AFB_1 (500-3,500 µg/kg), AFB_2 (2,000-4,000 µg/kg) and AFG_1 (1,000-4,000 µg/kg) until pupation showed a similar mortality across these treatments and a mortality increasing with higher doses (16-53%). Additionally, exposure to a combination of 3,500 µg AFB_1/kg and 75,000 µg kojic acid/kg resulted in an almost 9% increase in mortality compared to AFB_1 exposure alone (Sadek 1996). *Ostrinia nubilalis* Hübner (European corn borer) larvae, specialized on corn (*Zea mays* L.), showed a high tolerance towards AFB_1 exposure with a calculated median lethal concentration (LC_{50}) of 2,300 µg/kg diet (Mencarelli *et al.* 2013). The silkworm *B. mori* showed 100% mortality after 4 days of exposure to 15,614 µg AFB_1/kg (Ohtomo *et al.* 1975). Similarly, oral administration of 12,000 µg bassianolide/kg also was observed to be lethal to *B. mori* larvae after an exposure of 6 to 8 days (Kanaoka *et al.* 1978). Paterson *et al*. (1987) exposed *S. littoralis* larvae to a variety of mycotoxins, all at a concentration of 10,000 µg/kg. Highest mortality was observed after exposure to penicillic acid (90%) and brevianamide A (78%). Exposure to viomellein, OTA, cyclopenol, and citrinin led to 30%, 40%, 26%, and 48% mortality, respectively, indicating a varying susceptibility of *S. littoralis* larvae to different types of mycotoxins (Paterson *et al.* 1987). Exposure of *Spodoptera litura* Fabr. to 88-264 µg destruxin/kg body weight (*Metarhizium anisopliae* M-10 isolate) caused 30-90% mortality, after 48 hours. Destruxin obtained from a *Metarhizium anisopliae* M-19 isolate needed to be fed in nearly three times the doses to obtain the same percentage of mortality (Sree and Padmaja 2008). Exposure to 40,000-60,000 µg destruxin B/kg showed 7 to up to 30% mortality after 3 days, gradually increasing to 60-90% mortality after 8 days of exposure (Rizwan-Ul-Haq *et al.* 2009). Paterson *et al*. (1990) reared *S. frugiperda* larvae on substrates containing brevianamide A, brevianamide D and OTA up to a concentration of 10,000 µg/kg for 3 days and observed no mortality caused by brevianamide A and D. However, the observed mortality until pupation was considered significant for all treatments (Paterson *et al.* 1990). *Am. transitella* larvae seemed less sensitive to OTA exposure; concentrations of 1,000-50,000 µg OTA/kg for 12 days showed no significant difference in mortality and resulted in a 10% mortality in *Hel. zea* after exposure to 1,000 and 5,000 µg OTA/kg after 10 days (Niu *et al.* 2009). For both *S. frugiperda* and *Hel. zea* larvae exposed to 25,000 µg/kg dihydroxyaflavinine and roseotoxin B, a significantly higher mortality than the control was observed, while after exposure

to 2,500-25,000 µg penitrem A/kg a significant higher mortality (≥15%) was only observed in *Hel. zea* (Dowd *et al.* 1988).

Other orders

O. fasciatus larvae showed 100% mortality when exposed to 5,000 µg AFB_1/kg for 20 days, whereas a lower mortality was found after a shorter exposure time (Llewellyn *et al.* 1988). In honey bee *Apis mellifera* L. (Hymenoptera) exposed to 1,000 µg AFB_1/kg 30% mortality was observed (Figure 2d), while exposure to 15,000 µg AFB_1/kg caused 100% mortality after 60 hours of treatment (Niu *et al.* 2011). Exposure to 1,000 µg DON/kg did not affect survival in *Sitobion avenae* Fabr. nymphs, but caused 50% mortality in *Acyrthosiphon pisum* Harris nymphs. Mortality < 19% was observed for both species when exposed to 500-3,000 µg/kg deoxynivalenol-3-glucoside (DON3G) (De Zutter *et al.* 2016).

Accumulation and metabolism

Diptera

In the order of Diptera most studies on mycotoxin accumulation and metabolism were performed on *H. illucens* larvae. Concentrations observed were below the limit of quantification (LOQ) in *H. illucens* larval body when the larvae were given feed spiked with AFB_1 (8-390 µg/kg), DON (3,900-125,000 µg/kg), ZEN (280-13,000 µg/kg), or OTA (170-1,300 µg/kg) either as single mycotoxin or in mixtures with different concentrations of AFB_1, DON, ZEN, and OTA (Camenzuli *et al.* 2018). For the AFB_1 treatments (Figure 3a), only ≤18% of the initial concentration of AFB_1 present in the substrate was found in residual feed material and neither aflatoxicol (AFL), aflatoxin M_1 (AFM_1) nor aflatoxin P_1 (AFP_1) were detected in levels above the LOQ in the residual material (Camenzuli *et al.* 2018). When *H. illucens* larvae were fed a naturally contaminated diet containing AFB_1 (13.3 µg/kg), AFB_2 (2.6 µg/kg) and AFG_2 (7 µg/kg), 10.9 µg/kg AFB_1 and concentrations below the LOQ for AFB_2 and AFG_2 were detected in the residual material (Purschke *et al.* 2017). Exposure of *H. illucens* larvae to substrates containing AFB_1 as part of a mixture of mycotoxins (AFB_1, DON, ZEN, and OTA) in different concentrations resulted in a lower concentration found in the residual material compared to the initial concentration than when fed single mycotoxins. However, when fed with a mixed diet containing 430 µg AFB_1/kg, 100,000 µg DON/kg, 9,400 µg ZEN/kg and 2,000 µg OTA/kg a small amount of AFL was also formed (Camenzuli *et al.* 2018). A higher percentage of the initial concentration in the substrate was found back in the residual material of *H. illucens* larvae when DON was added as part of a mixture containing also AFB_1, ZEN, and OTA (Camenzuli *et al.* 2018). When *H. illucens* larvae were exposed to a substrate with an initial concentration of 170-1,700 µg OTA/kg, 41-62% was found back in the residual feed substrate (Camenzuli *et al.* 2018). Correspondingly, feed spiked with concentrations of AFB_1, DON, OTA, and ZEN either within the same range or lower than Camenzuli *et al*. (2018) resulted in non-detectable levels in *H. illucens* larvae (Purschke *et al.*

2017) and levels below the detection limit (LOD, limit of detection) when fed with 415 µg/kg AFB_1 (Bosch *et al.* 2017). In a study performed by Leni *et al*. (2019) concentrations below the LOD were observed in the larval body of *H. illucens* fed on naturally contaminated substrates containing 779 µg/kg DON, 573 µg/kg FB_1, and 441 µg/kg FB_2 (Leni *et al.* 2019). It must be noted that data obtained for *H. illucens* larvae might not representative for all species within the order, since one study observed a 10-fold higher concentration of AFB_1 in 2nd instar *Musca domestica* L. larvae after only two days of exposure to 20 µg/kg AFB_1 (Nevins and Grant 1971).

Coleoptera

Several studies investigating mycotoxin accumulation for species in the order Coleoptera were found, nevertheless they only focus on *T. molitor* and *A. diaperinus*.

In 1st instar *T. molitor* larvae fed with 13 µg/kg AFB_1, concentrations in the larval body were below the LOD. However, AFB_1 was detected at 1% and 10% of the EC legal limit (being 20 µg/kg, Directive 2002/32/EC) after being fed with 23 or 415 µg/kg AFB_1, respectively (Bosch *et al.* 2017). As shown in Figure 3a, the percentage of AFB_1 found back in the residual feed material of *T. molitor* larvae was low and seems to be dependent on the initial AFB_1 concentration in the feed. In the residual feed material of *T. molitor* larvae, formation of small amounts of AFM_1 were found, however, it must be noted that other AFB_1 metabolites were not quantified in this study (Bosch *et al.* 2017).

As described for AFB_1, also levels of DON were below the LOD in the larval body of *T. molitor* when exposed to up to 12,000 µg/kg DON (Niermans *et al.* 2019; Ochoa Sanabria *et al.* 2019; Van Broekhoven *et al.* 2017). However, the concentration of DON found in the residual feed material varied between studies. Van Broekhoven *et al*. (2017) showed that the percentage of excreted DON was lower in the residual feed material of larvae fed a naturally contaminated diet (*ca.* 14%) as opposed the percentage found in the residual feed material of larvae fed a spiked diet (*ca.* 41%; Van Broekhoven *et al.* 2017). A second study found only a minor difference between the percentage of excreted DON when fed a spiked (58%) or a naturally contaminated (46-52%) diet after eight weeks of exposure (Niermans *et al.* 2019). The percentage of excreted DON in *T. molitor* varied between 6.2-16.2% and appeared to decrease when fed with increasing concentrations, and the metabolite 3-acetyldeoxynivalenol (3AcDON) was detected in the residual feed material in concentrations which were similar for all diets fed (Ochoa Sanabria *et al.* 2019). Since 3AcDON was only chemically analysed in residual feed materials of *T. molitor*, its presence cannot be compared with other species. Another study included the DON derivatives DON3G and 15-acetyldeoxynivalenol (15AcDON) in the analyses, but did not find them in the residual material (Van Broekhoven *et al.* 2017).

Similar trends were observed in *T. molitor* larvae when exposed to ZEN (Table S3). Levels detected in the larval body of *T. molitor* were below the LOD/LOQ when exposed to

concentrations up to 2,283 µg/kg ZEN. The percentage of excreted ZEN was lower in the residual feed material of larvae fed a naturally contaminated diet (19-23%) as opposed to the percentage found in residues of larvae fed a spiked diet (56-63%). As shown in Figure 3b, formation of α- and β-zearalenol in different concentrations was observed in all diets fed (Niermans *et al.* 2019).

The study of Piacenza *et al*. (2020) showed that no T-2, HT-2, T-2 triol or T-2 tetraol were detected (in levels above the LOD) in the surviving larvae or in the dead larvae collected from the control diets. However, after examination of the dead larvae collected from the other diets, 44.2 µg/kg T-2 was found in the body of the larvae fed with the naturally contaminated diet (250 µg/kg total T-2 and HT-2) and 7.7 µg/kg T-2 in the larvae fed the naturally contaminated diet (100 µg/kg total T-2 and HT-2) and the artificially contaminated diets. Additionally, T-2 and HT-2 were found in the residual material of both the naturally and artificially contaminated diets (except for the controls). The percentage of excreted T-2 found in the residual materials were higher when fed a naturally contaminated diet (51.7-66.5%) as opposed to an artificially contaminated diet (36.5-55.1%). HT-2 was only observed in the residual feed material of the larvae fed the artificially contaminated diets, interestingly it seemed that the concentration found was not affected by the initial dose (Piacenza *et al.* 2020).

Currently, no data are available on the accumulation or reduction of OTA in *T. molitor* residues. Data on FB_1 are only available for *T. molitor* larvae and showed that when fed with doses varying between 50,000-450,000 µg FB_1/kg approximately 40% of the initial concentration was found back in the residual feed material. Accumulation within the larval body was not discussed and metabolites were not included in this study (Abado-Becognee *et al.* 1998). Corresponding to what was found in *T. molitor* larvae, concentrations lower than the LOD/LOQ were observed in *A. diaperinus* larvae fed with substrates containing 8-390 µg/kg AFB_1, 3,900-125,000 µg/kg DON, 280-13,000 µg/kg ZEN, or 170-1,300 µg/kg OTA tested alone and after exposure to combined mycotoxin concentrations up to 100,000 µg DON/kg (Camenzuli *et al.* 2018), 727 µg/kg FB_1, and 294 µg/kg FB_2 (Leni *et al.* 2019).

The study of Camenzuli *et al.* (2018) showed that in the residues of *A. diaperinus* larvae, AFM_1 seems to be the main metabolite and AFL is also formed at the highest concentration (390 µg AFB_1/kg). The authors also included the metabolites AFP_1 and aflatoxin Q_1 in their study, but the concentrations found were below the LOQ (5 µg/kg). *A. diaperinus* larvae were exposed to substrates containing AFB_1 as part of a mixture of mycotoxins (AFB_1, DON, ZEN and OTA) in different concentrations. When compared with single AFB_1 exposure of *A. diaperinus* larvae, the concentration found in the residual material was lower as compared to the initial concentration as when fed single mycotoxins. Also, formation of the same metabolites was observed. The percentage of excreted DON was similar when fed as single compound and when fed as part of a mixture and ranged between 80-96%. Concentrations of the included DON metabolites (3AcDON, 15AcDON and DON3G) were below their respective LOQ´s (Camenzuli *et al.* 2018).

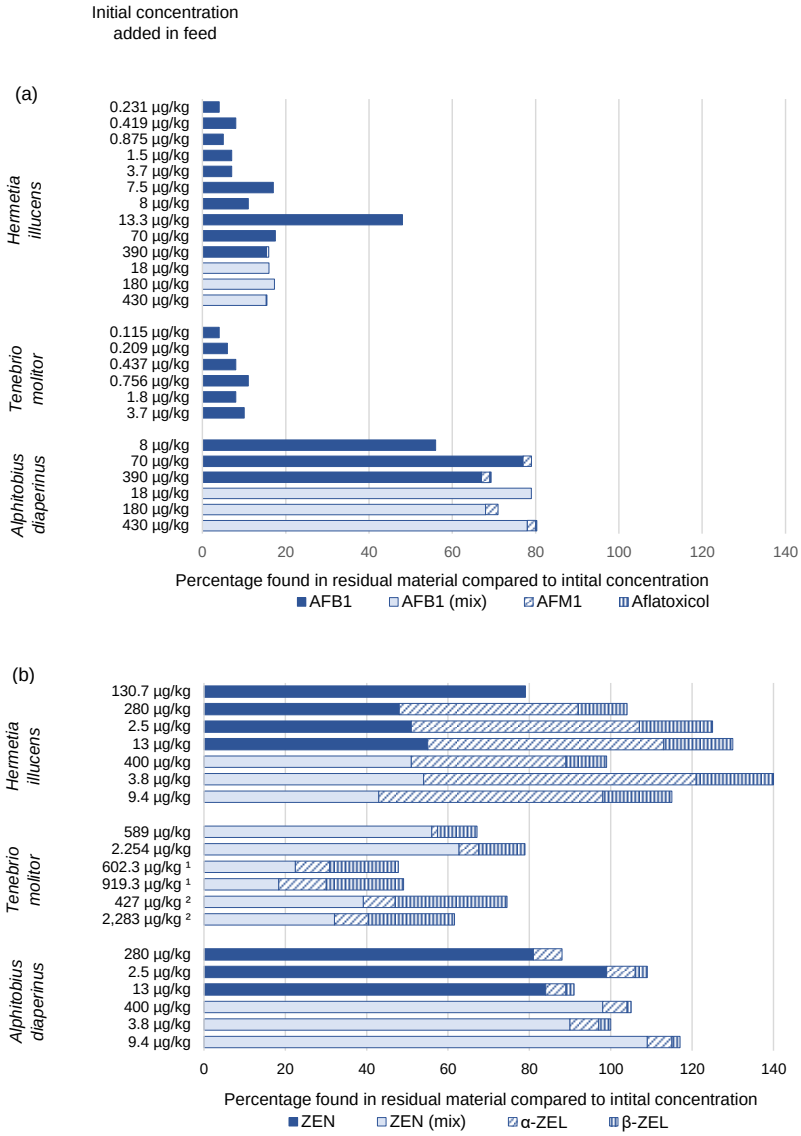

Figure 3. Overview of mycotoxin concentration in the feed substrate residues relative to the initial concentration (µg/kg) present in the feed substrate in percentages for *H. illucens*, *T. molitor*, and *A. diaperinus.*

[1]Naturally contaminated, [2]Artificially contaminated, α-ZEL: α-zearalenol, β-ZEL: β-zearalenol, when not specified, the mycotoxins are spiked to the initial substrate. In none of the included studies, concentrations above the LOQ/LOD were found in the larvae, therefore these percentages only represent the concentration found back in the residual material compared to the concentration in the initial substrate. In some of the studies the final amount of residue was not mentioned. Based on the studies of, a: Bosch *et al*. (2017), Camenzuli *et al*. (2018), Purschke *et al*. (2017); b: Camenzuli *et al*. (2018), Niermans *et al*. (2019), Purschke *et al*. (2017). An overview of the data used are available in Table S3.

In residual feed material of *A. diaperinus* larvae, the percentage of α-zearalenol (α-ZEL) formed is similar when fed with initial concentrations ranging from 280–13,000 µg ZEN/kg. β-zearalenol (β-ZEL) was not formed, when an initial concentration of 280 µg/kg ZEN was fed to the larvae, and seems to be formed only at higher initial concentrations in the feed (Figure 3b). Exposure to a mycotoxin mixture, resulted in a similar reduction of the concentration found in the residual material compared to the initial concentration of ZEN and formation of α-ZEL and β-ZEL in *A. diaperinus* as when spiked with ZEN alone (Camenzuli *et al.* 2018). For *A. diaperinus* larvae fed an OTA containing substrate (initial concentration of 170-1,700 µg OTA/kg), 97-115% was found back in the residual material. Comparable results were found when OTA was fed as part of a mycotoxin mixture (AFB_1, DON and ZEN) (Camenzuli *et al.* 2018).

Lepidoptera

Only one study investigated mycotoxin accumulation in species of the order Lepidoptera, including *Hel. zea* and *O. nubilalis* larvae reared on a diet containing 5,000 µg/kg ZEN. ZEN was not detected in 4-day old *O. nubilalis* larvae whereas 650 µg ZEN/kg was observed in *Hel. zea* larvae of the same age. After 7 days of feeding, ± 600 µg ZEN/kg was also detected in *O. nubilalis* larvae. However, over time, a constant decrease of ZEN was observed in the larval body of both species. Feed residues were not analysed in this study (Bily *et al.* 2004).

Other orders

When *P. americana* were fed with 12,000 µg AFB_1/kg detectable levels up to 2 µg AFB_1/kg were found in 40% of the tested insects, residual feed materials were not analysed in this study (Llewellyn *et al.* 1976)

Enzymes responsible for insect mycotoxin biotransformation

An explanation for the unrecovered fraction of mycotoxins could be that mycotoxin biotransformation occurs in the insects, therefore the following section contains an overview of all studies suggesting enzymes responsible for mycotoxin biotransformation in insects (Table 2). The authors are not aware of any studies covering possible responsible enzyme systems in Coleoptera.

Diptera

Few studies regarding the metabolization of mycotoxins are available for *H. illucens* and *D. melanogaster*. Meijer *et al*. (2019) used an *H. illucens* S9 fraction, in combination with the cytochrome P450 (CYP450)-enzyme inhibitor piperonyl butoxide (PBO) and showed that CYP450s were responsible for the metabolic conversion of AFB_1 into AFP_1 and pointed to a role of a cytoplasmic reductase for conversion of AFB_1 into AFL (Meijer *et al.* 2019). In addition, the CYP450-enzyme CYP6A2 originating from the Oregon-R(R) strain of the fruit fly, *D. melanogaster*,

seemed to be at least partially responsible for bioactivation of AFB_1 to a recombinagen in a *Saccharomyces cerevisiae* strain, however, this metabolic activity seemed to be dependent on co-expression with a human-derived NADPH-CYP450-oxidoreductase (Saner *et al.* 1996).

Table 2. Overview of suggested systems involved in mycotoxin metabolism in different insect species

Species	Mycotoxin	Enzyme system involved in mycotoxin metabolism and resulting metabolite if reported	Reference
Diptera			
Hermetia illucens	AFB_1	CYP450 (AFP_1); Cytoplasmic reductase (AFL) [1]	Meijer *et al*. (2019)
Drosophila melanogaster (strain Oregon R(R))	AFB_1	CYP450 (CYP6A2) [8,9], depended on co-expression with a NADPH-CYP450-oxidoreductase	Saner *et al*. (1996)
Lepidoptera			
Helicoverpa zea	AFB_1	CYP450 [3]	Zeng *et al*. (2006)
Helicoverpa zea	AFB_1	CYP450 (CYP321A1; AFP_1) [1,4,5]	Niu *et al*. (2008)
Helicoverpa armigera	AFB_1	CYP450 (CYP6AE19) [4,7]	Elzaki *et al*. (2019)
Amyelois transitella	AFB_1	CYP450 (AFB_{2a}, AFM_1); NADPH-dependent reductase (AFL) [5]	Lee and Campbell (2000)
Trichoplusia ni	AFB_1	CYP450 [4]	Zeng *et al*. (2013)
Other			
Apis mellifera	AFB_1	CYP450 [3]	Niu *et al*. (2011)
Apis mellifera	AFB_1	CYP450 (three CYP6AS) [8]	Johnson *et al*. (2012)
Acyrthosiphon pisum	DON	Glucosyltransferase (DON-3G) [1]	De Zutter *et al*. (2016)
Sitobion avenae			

Methods of measurements: [1] LC-MS/MS, [2] Spectrophotometric enzyme assay, [3] bioassays, [4] RT-PCR, [5] HPLC, [6] Enzyme assays, [7] gene-silencing, [8] northern blotting, [9] southern blotting. CYP450: cytochrome P450s

Lepidoptera

ABF_1 metabolism was not observed in midgut enzyme isolates from *Hel. zea* larvae fed with a control diet. However, AFB_1 metabolism was observed in midgut isolates from larvae grown on diets supplemented with either coumarin or xanthotoxin in which the relatively non-toxic AFP_1 was the main metabolite identified. Additionally, AFP_1 formation was completely inhibited by the addition of PBO, required NADPH and therefore indicated the role of CYP450s and more specifically CYP321A1 (Niu *et al.* 2008). The role of CYP450s in bioactivation of AFB_1 in *Hel. zea* was identified after performance of a series of bioassays in which AFB_1 toxicity was assessed in the presence of PBO (inhibitor) and phenobarbital (inducer). Addition of PBO caused a significantly decreased toxicity and an increased pupation rate in 4th and 5th instar *Hel. zea* larvae

(Zeng *et al.* 2006). Corresponding to what was mentioned previously, also a second study showed that PBO reduced the toxicity of AFB_1 to 5-day-old *Tr. ni* larvae (Zeng *et al.* 2013). When *H. armigera* larvae were fed a diet containing AFB_1 and injected with ds*CYP6AE19* to silence *CYP6AE19* expression, a decreased mortality was found compared to when fed the same diet but injected with ds*GFP* (green fluorescent protein) or water suggesting that induction of CYP6AE19 results in a higher toxicity of AFB_1 (Elzaki *et al.* 2019). In an *in vitro* inhibition study of AFB_1 metabolism, larvae of *Am. transitella* produced AFB_{2a}, AFM_1 and mostly AFL, while for *Cydia pomonella* only AFL was detected at trace level. After adding PBO, production of AFB_{2a} and AFM_1 by *Am. transitella* was completely inhibited, indicating a role for CYP450s. Additionally, the role of NAD(P)H and glutathione (GSH) in AFL production was tested and showed that NADPH and GSH were equally effective in AFL production in *Am. transitella*, whereas a mixture of both enhanced AFL production. The authors concluded that a NADPH-dependent reductase seems to be responsible for the transformation of AFB_1 into AFL and suggest the involvement of GSH as an electron donor in AFL formation (Lee and Campbell 2000).

Other orders

The role of a phase II enzyme glucosyltransferase was proposed to be involved in the detoxification of DON into DON3G in the aphids *Si. avenae* and *Ac. pisum.* Interestingly, *Si. avenae*, which co-occurs with the DON-producing *Fusarium graminearum* converted DON to DON3G more efficiently than *Ac. pisum* which normally feeds on plants not considered as host for *F. graminearum* (De Zutter *et al.* 2016). It was hypothesized that natural phytochemicals which are present in the insects' food could possibly induce CYP450 activity and help in the detoxification of AFB_1. To support this statement the effect of AFB_1 in combination with honey was examined and it was observed that *A. mellifera* adults fed on honey were more tolerant to AFB_1 exposure than bees fed on other diets. An elevated expression of three *CYP6AS* CYP450 genes were observed in northern blot analyses of the guts of bees fed extracts of honey, pollen and propolis and suggested that consumption of possible phytochemicals present in honey can induce CYP450s responsible for detoxification of AFB_1 in *A. mellifera* (Johnson *et al.* 2012). In addition, a role for CYP450s was indicated in AFB_1 detoxification in *A. mellifera*. A decreased survival time was observed in *A. mellifera* after consumption of bee candy containing 10,000 µg/kg AFB_1 supplemented with either 0.05% PBO or 0.1% PBO as compared to when bee candy with the same concentration of AFB_1 was consumed alone. Additionally, the authors fed *A. mellifera* with two concentrations of OTA (10,000 and 40,000 µg/kg) supplemented with either 0.05% PBO or 0.1% PBO, but in this case the addition of PBO did not seem to affect survival time (Niu *et al.* 2011).

Discussion

Tolerance

The insect sector offers potential to promote circular and sustainable opportunities for feed production. Also the contribution to organic waste management is interesting from an economic and ecological point of view. When using waste- or side streams as a substrate for insect rearing low-quality streams can be upgraded into high-quality protein- or fat fractions. Another ecological advance supported by this review is that insects can breakdown complex mycotoxins and metabolize them into smaller less- or non-toxic metabolites. However, it needs to be clarified whether insects fed on these possibly contaminated waste- or side streams show a high tolerance and yield enough biomass for economically feasible production.

This systematic review summarized published data about tolerance to mycotoxins for insect species mainly belonging to three insect orders. Although the available information is rather limited, tolerance differences between orders, within orders and even between strains and stages of the same species becomes apparent (Figure 2a). In addition to differences between insect species, growth and mortality were affected differently by the type of mycotoxin, the concentration of the mycotoxin fed and the life-stage in which mycotoxin exposure occurred. The studies on Coleoptera showed a reduction as well as an increase in biomass after exposure to mycotoxin-contaminated diets. The actual effect depended on the mycotoxin and its concentration and whether the toxin was present naturally or spiked to the substrate. Growth effects caused by mycotoxin exposure of Lepidoptera were reported in several studies and showed clear variation in tolerance between species, larval stages, and mycotoxins. Overall, insects of the order Coleoptera show lower mortality after exposure to AFB_1 when compared to Lepidoptera and Diptera. Additionally, the inclusion of certain supplements in the mycotoxin-contaminated diet showed to have an influence as exposure of *Tr. ni* larvae to a diet containing the plant allelochemical xanthotoxin in combination with AFB_1 resulted in a substantially higher weight and pupation rate when compared to AFB_1 exposure alone (Zeng *et al.* 2013).

All papers which discussed the effect of mycotoxin exposure on insects are included in this review. In some papers the application of mycotoxins for biocontrol of insects is the main focus. These papers included mycotoxins such as roseotoxin B and brevianamides which are currently not considered as food-relevant. However, the future aim is to use (contaminated) organic waste- and/or side streams as substrate for insect rearing, rather than substrates from a solely food-relevant origin. Some of these mycotoxins are relevant for biocontrol of insects, and therefore might affect insect tolerance and/or growth. As discussed in this review, exposure of *S. littoralis* larvae to 10,000 µg/kg penicillic acid or brevianamide A led to a mortality of 90% and 78% respectively (Paterson *et al.* 1987), which suggests that substrates containing these toxins in a similar or higher concentration might be unsuitable for insect rearing. Overall, the data

discussed provides a positive outlook for the use of mycotoxin-contaminated organic waste- and/or side streams in the future.

Type of substrate

Substrate materials investigated in the retrieved studies ranged from poorly defined waste streams to refined sugar. The type of substrate fed could have had an effect on the study outcome, as was shown in the case where mortality of *A. mellifera* exposed to AFB_1 in honey was lower than when fed with AFB_1 in sucrose (Johnson *et al.* 2012). In the retrieved studies, insects were exposed via artificially contaminated, spiked and naturally contaminated substrates, resulting in heterogenous effects. *T. molitor* larvae reared on wheat naturally contaminated with mycotoxins gained significantly more weight than when reared on spiked or artificially contaminated wheat (Niermans *et al.* 2019; Van Broekhoven *et al.* 2017). Naturally contaminated substrates might contain a mixture of mycotoxins or modified forms that could have had a synergistic effect on the larvae when exposed to them, which offers the insect a very different situation as compared to being fed a substrate spiked with a single mycotoxin. Accordingly, the studies included in this review showed that the presence of multiple mycotoxins in the insect diet, compared to the presence of a single mycotoxin, influenced mortality as was shown when *Hel. zea* larvae fed on a substrate contaminated with fusaric acid and the plant secondary metabolite gossypol experienced 18% mortality as opposed to fusaric acid or gossypol alone not leading to mortality (Dowd 1988). The results obtained from feeding studies in which the substrate was spiked with a single mycotoxin may therefore be not fully representative for when waste- or side streams are used. However, using spiked feed is more controlled and is necessary for a first exploration of the effects of single mycotoxins on insect tolerance and metabolism.

Accumulation/biotransformation

When insects are sold for human or animal consumption, they need to comply to the MLs and GVs set in the respective legislations, which makes no/low mycotoxin accumulation an important requirement. Mycotoxin accumulation in the insect body was observed in some specific cases, however, the concentration in the insects found was mostly below their respective MLs or GVs. In general, the available data demonstrate that mycotoxin levels in the insect larvae are below the respective LOD/LOQ, even when exposed to concentrations above the European Commission ML for the presence of mycotoxins in food and their commodities, and GVs set for mycotoxins in feed (EC 2002a; EC 2006b). Additionally, the available studies have shown that only a fraction of the initially added mycotoxin concentration in the feed was found back in the residual feed material (Figure 3a), even when taking into account main metabolites that could be formed. The unrecovered fraction could be explained in multiple ways; interference by the matrix leading to a loss of signal, breakdown of parent compounds, transformation into modified forms and/or the formation of unknown metabolites, amongst others. The unrecovered fraction

was larger for DON, OTA, and AFB_1 in *H. illucens* and *T. molitor* larvae as compared to *A. diaperinus* larvae. Formation of the toxic metabolite α-ZEL was highest in the residual feed material of *H. illucens* when compared to the concentrations found in the residues of *T. molitor* and *A. diaperinus* (Figure 3b) (Camenzuli et al. 2018). Identification and quantification of unknown metabolites will allow for a more complete mass balance in the future and will give a better insight in the possible detoxification by insects.

Enzyme systems

Insects have developed metabolic adaptations that can result in detoxification and/or yield metabolites that are easier to transport or excrete (Birnbaum and Abbot 2018). Metabolites formed can be more or less toxic than the parent compound (in this case the original mycotoxin in the substrate), and they should be identified and investigated for toxicity to ensure safety when insects are used as food or feed ingredients. Identification of enzyme systems responsible for the formation of mycotoxin metabolites will foster insights in the pathways involved in mycotoxin metabolism in the insect body and, hence, the possible metabolites that are formed. To date, most studies that identified enzyme systems involved in mycotoxin metabolism mainly focused on AFB_1 (Table 2). In insects, enzymes for phase I metabolism, CYP450s, as well as phase II enzymes, glycosyltransferases, are known to transform AFB_1. A recently published review covering CYP450-mediated mycotoxin metabolism in plant-feeding insects concluded that the involved CYP450s mostly belong to families known to detoxify phytochemicals (Berenbaum *et al.* 2021), which is in accordance with data found in this review. CYP450s were involved in the conversion of AFB_1 to mostly AFP_1 and the roles of GSH and NADH were identified in the formation of AFL in *Am. transitella*. CYP450 enzymes and their subfamilies are also found in most tissues of various animal species where they play a role in mycotoxin metabolism. (Hussein and Brasel 2001). As an example, chicken and quail hepatic microsomes use CYP2A6 and to a lesser extent CYP1A to transform AFB_1 into the extremely reactive AFB_1-8-9-epoxide (Diaz *et al.* 2010). A cytosolic reductase important in the reduction of AFB_1 to AFL seems to be produced in poultry as well, however, in larger quantities in turkey and duck then in quail and chicken (Peles *et al.* 2019). In bovine hepatocytes AFM_1, mainly formed by CYP1A and CYP3A hepatic monooxygenase activities, seemed to be the most prominent metabolite formed within the first hours of incubation (Kuilman *et al.* 2000). Additional to the shared importance of the CYP450 enzymes, cytoplasmic reductases and GSH, no solid conclusions can as yet be made on the comparison between the metabolism of mycotoxins by insects and another animals.

Other uncertainties

The LOD of the analytical system used to determine mycotoxin concentrations in the substrates, larval material and the residues is a critical point in this discussion. Although Table S1 gives an extensive overview of all analytical methods used, the relevant LODs are not always known. A weak analysis can result in a no-toxin level in the larvae, while in reality the method or machine

used might have a limited sensitivity. A sensitive method of analysis is especially important for measuring the presence of aflatoxins since very low concentrations are already unwanted when insects are used for used for food and/or feed purposes later on (Table 1). However, most recent studies do provide information on the sensitivity and detection limits of the analyses performed and are able to detect mycotoxin concentrations in insects in levels far below their respective legal limits (Camenzuli *et al.* 2018; Meijer *et al.* 2019). Finally, the question remains whether the entire amount of feed (and therefore the present toxin) was consumed by the insects during the exposure period and how this, when not fully consumed, would affect the data obtained in the discussed studies.

Outlook

This study presents comprehensive data on the effects of mycotoxins on insect growth and survival, as well as mycotoxin accumulation and conversion by insects. Most data relate to species which are agricultural pests and species potentially used as food or feed in the EU. Survival and growth as well as tolerance and metabolization varies between species, between mycotoxins, and their concentration as well as the type of substrate used, whether the mycotoxin was present naturally or spiked, and the presence of possible supplements. Accumulation of mycotoxins was identified as mostly below LOD/LOQ for the included species. Since data cannot be generalized across species and not even across strains of the same species, additional studies on other insect species than the main species covered in this review (*H. illucens*, *T. molitor* and *A. diaperinus*) are recommended, specifically on insects possibly considered for food/feed including crickets and locusts. CYP450s were suggested as main enzymes involved in AFB_1 metabolism in some insects, however further research is recommended on unravelling metabolic pathways, involvement of Phase II enzymes, the formation of possible unknown metabolites and their toxicity. Overall, based on the available data, the use of mycotoxin-contaminated waste streams as substrate for insect rearing seems to provide a promising approach for the future of mycotoxin remediation and a circular economy.

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Supplementary materials

Table S1

Overview of covered studies including: insect species, substrate used, exposure time of the insects, analytical method used, and mycotoxins (metabolites) analysed

Reference	Orders	Species	Substrates	Insect stage	Time of exposure	Method of measurement	Mycotoxins Analysed [1]	Mycotoxin concentrations in feed	Fungus present
Abado-Becognee *et al*. (1998)	Coleoptera	*Tenebrio molitor*	Maize flour with 4% (w/w) mineral salt and 13% vitamin B complex	Larvae (7 weeks)	63 days	HPLC w Fluorescence Detector	FB_1	FB_1: 50,000, 150,000, 450,000 µg/kg	No
Bily *et al*. (2004)	Lepidoptera	*Helicoverpa zea* and *Ostrinia nubilalis*	*Hel. zea*: soybean flour/wheat germ artificial diet. *O. nubilalis*: Meredic diet	Larvae (3rd instar)	4, 7, 11 and 14 days	LC-MS	ZEN	ZEN: 5,000 µg/kg	No
Bosch *et al*. (2017)	Coleoptera, Diptera	*Tenebrio molitor* and *Hermetia illucens*	Poultry feed	Larvae (1st instar)	*T. molitor*: 40 days; *H. illucens*: 10 days	HPLC w Fluorescence Detector	AFB_1, AFM_1	AFB_1: 10, 25, 50, 100, 250 and 500 ug/kg	No

Camenzuli *et al*. (2018)	Coleoptera, Diptera	*Alphitobius diaperinus* and *Hermetia illucens*	*A. diaperinus:* wheat based with apple; *H. illucens:* wheat based and water	Larvae (*A. diapernius*: 2 weeks and *H. illucens*: 1 week)	*A. diapernius*: 14 days; *H. illucens*: 10 days	LC-MS/MS	AFB_1, AFL, AFM_1, AFP_1, AFQ_1; DON, 3AcDON, 15AcDON, DON3G; ZEN, α-ZEL, β-ZEL and OTA	AFB_1: 8, 70 and 390 µg/kg; DON: 3,900, 38,000 and 112,000 µg/kg; ZEN: 280, 2,500 and 13,000 µg/kg; OTA: 170, 1,700 and 1,300 µg/kg. Mixtures: AFB_1: 18, 180 and 430 µg/kg; DON: 4,100, 41,000 and 100,000 µg/kg ; ZEN: 400, 3,800 and 9,400 µg/kg; OTA: 80, 800 and 2,000 µg/kg	No
Cao *et al*. (2019)	Diptera	*Drosophila simulans*	Grapes	Eggs-adult	Until adult	Not mentioned	OTA	OTA: 0.05, 0.5 µg/kg	Yes
Chinnici *et al*. (1979)	Diptera	*Drosophilia melanogaster* (strains A-9 and A-11)	Control media and media containing AFB_1 (prepared according to Chinnici et al. 1976)	Larvae (1-5 days)	Until adult	Thin layer chromatography	AFB_1	AFB_1: 440 and 880 µg/kg	No

Cito *et al*. (2016)	Coleoptera	*Tenebrio molitor*	Bread and bran	Larvae (mature)	20 days	LC-UV-MS	Beauvericin A, bassianolide and beauverolides	Not specified	*Beauveria bassiana* (strains: B 13/I03, B 13/I11, B 13/I49, B 13/I57, B 13/I63, and B 13/I64)
Davis and Schiefer (1982)	Coleoptera	*Tenebrio molitor*	Whole wheat supplemented with brewer's yeast (9:1)	Larvae (8.5-11.5 mg)	4 weeks	Calculations from known concentration	T-2	T-2: 2,000, 4,000, 8,000, 16,000, 32,000, 64,000 and 128,000 µg/kg	No
De Zutter *et al*. (2016)	Hemiptera, Homoptera	*Sitobion avenae, Acyrthosiphon pisum*	Wheat seedlings and young broad bean plants	Nymphs	3 days	LC-MS/MS	DON, DON3G, 3- and 15AcDON	DON: 500, 1,000, 3,000 µg/kg; DON3G: 500, 1,000, 3,000 µg/kg	No
Dowd (1989)	Lepidoptera	*Heliothis zea*	Pinto bean-based diet + three allelochemicals (gossypol, a saponin, and 6-methoxy2-benzoxazolinone)	Larvae (neonate)	7 days	Calculations from known concentration	Fusaric acid	Fusaric acid: 25,000 and 250,000 µg/kg	No
Dowd (1990)	Lepidoptera	*Heliothis zea, Spodoptera frugiperda*	Pinto bean	Larvae (last instar)	48 hours	Thin layer chromatography	DON, T-2, diacetoxyscirpenol	DON: 2,500, 25,000, 250,000 µg/kg; T-2: 2,500, 25,000 µg/kg; diacetoxyscirpenol: 2,500, 25,000 µg/kg	No

Dowd (1993)	Lepidoptera	*Helicoverpa zea, Spodoptera frugiperda*	Pinto bean	Larvae (1st and 3rd instar)	7 days	HPLC	Griseofulvin	Griseofulvin: 25,000 µg/kg	No
Dowd *et al*. (1988)	Lepidoptera	*Heliothis zea, Spodoptera frugiperda*	Pinto bean	Larvae (neonate)	7 days	Not mentioned	Dihydroxyaflavinine, roseotoxin B, penitrem A, verruculogen, cytochalasin H, paspaline, paxilline	Up to 25,000 µg/kg ww (100,000 µg/kg dw) of dihydroxyaflavinine, roseotoxin B, penitrem A, verruculogen, cytochalasin H, paspaline or paxilline	No
Elzaki *et al*. (2019)	Lepidoptera	*Helicoverpa armigera*	Artificial diet + and - MeJA (prepared according to Qi et al. 2000)	Larvae (2nd instar)	Until pupation	Not mentioned	AFB_1	AFB_1: 1,000 µg/kg	No
Gulsunoglu *et al*. (2019)	Diptera	*Hermetia illucens*	SSF kernels	Larvae (2nd instar)	12 days	LC–MS/MS,	DON	DON: 630-3,580 µg/kg	*Fusarium* spp.
Gunst *et al*. (1982)	Diptera	*Drosophilia melanogaster* (strains Florida-9 and Lausanne-S)	Five types: control, media + AFB_1, media + AFB_1, media + AFG_1 and media + ST (prepared according to Chinnici et al., 1976)	Egg-adult	Whole life-cycle	Thin layer chromatography	AFB_1, AFB_2, AFG_1; sterigmatocystin	AFB_1, AFB_2, AFG_1; sterigmatocystin: 200, 600, 2,000 and 4,000 µg/kg	No

Hegde *et al.* (1967)	Lepidoptera	*Corcyra cephalonica*	Wheat bran, groundnut meal	Larvae (10 days)	20 days	Paper chromatography	AFB_1, AFG_1	AFB_1: 1,000,000 µg/kg	*Aspergillus flavus, Aspergillus oryzae, Penicillium purpurogenus* and *Penicillium rubrum*
Jankovic-Tomanic *et al*. (2019)	Coleoptera	*Tenebrio molitor*	Wheat bran artificially contaminated	Larvae (2 months)	2 weeks	Calculations from known concentration	DON	DON: 4,900, 8,000, 16,000 and 25,000 µg/kg	No
Johnson *et al*. (2012)	Hymenoptera	*Apis mellifera*	Bee candy (2 sucrose:1 water)	Newly emerged bees	3 days	Calculations from known concentration	AFB_1	AFB_1: 20,000 µg/kg	No
Kanaoka *et al*. (1978)	Lepidoptera	*Bombyx mori*	Artificial diet	Larvae (3rd instar)	8 days	Silica gel column chromatography	Beauvericin, bassianolide	Beauvericin: 4,000, 8,000, 1,000,000 µg/kg; bassianolide: 1,000, 2,000, 4,000, 8,000, 12,000 or 16,000 µg/kg	*Beauveria bassiana, Verticillium lecanii*
Kirk *et al*. (1971)	Diptera	*Drosophilia melanogaster* (strain Oregon R)	Yeast-corn meal molasses growth medium	Larvae (1st instar)	24 days	Not mentioned	AFB_1	AFB_1: 10,000 µg/kg	No
Lee and Campbell (2000)	Lepidoptera	*Amyelois transitella, Cydia pomonella*	*Am. transitella*: walnuts; *C. pomonella*: apples	Larvae (1st instar)	Not applicable	HPLC w Fluorescence Detector	AFB_1, AFB_2, AFB_{2a}, AFL, AFM_1, AFM_2, AFG_1, AFG_2, AFB_1-8,9-epoxide and AFB_1-8,9-epoxide-GSH conjugate	AFB_1: 3,123,000 µg/kg	No

Leni *et al*. (2019)	Coleoptera, Diptera	*Alphitobius diaperinus, Hermetia illucens*	Naturally contaminated by-products of corn, wheat, rice, rapeseed, apple, olive and carrots	Larvae (2 days)	*A. diaperinus*: 28 days; *H. illucens*: 15 days	HPLC w Fluorescence Detector, UHPLC-MS/MS	DON, 3AcDON, fusarenone X, diacetoxyscirpenol, T-2, HT-2, FB_1, FB_2, ZEN, AFB_1, patulin, OTA, nivalenol	DON: 416, 468, 557, 608, 726, 755 µg/kg; FB_1: 127 µg/kg; FB_2: < LOD; ZEN: < LOD	No
Llewellyn *et al*. (1988)	Hemiptera	*Oncopeltus fasciatus*	Water, unsalted raw sunflower seeds	Larvae (5th instar)	21 days	Calculations from known concentration	AFB_1	AFB_1: 5,000 µg/kg	No
Llewellyn *et al*. (1976)	Blattodea	*Paradelphomyia americana*	Sucrose water	Adult (males)	56 days	Not mentioned	AFB_1	AFB_1: 12,000 µg/kg	No
Matsumura and Knight (1967)	Diptera	*Aedes aegypti, Drosophila melanogaster* and *Musca domestica*	*Ae. aegypti*: distilled water; *D. melanogaster*: standard food medium; *M. domestica*: sucrose solution and milk (1:1)	*Ae. aegypti*: larvae (4th instar) and adults; *D. melanogaster*: adults; *M. domestica*: adults	5 days	Not mentioned	AFB_1	AFB_1: *Ae. aegypti* larvae 3,000 µg/kg (unclear for *Ae. aegypti* adults, *D. melanogaster* and *M. domestica*)	No
Meijer *et al*. (2019)	Diptera	*Hermetia illucens*	Wheat based mashed feed	Larvae (1st instar)	9 days	LC-MS	AFB_1	AFB_1: 500 µg/kg	No
Melone and Chinnici (1986)	Diptera	*Drosophilia melanogaster* (strains Oregon-R and Lausanne-S)	Fly culture medium (dextrose, yeast, agar, inorganic salts, and methyl p-hydroxybenzoate)	Egg-adult	Whole life-cycle, multiple generations	Calculations from known concentration	AFB_1	AFB_1: 500, 1,000, 1,300, 1,600, 1,900, 2,200 and 2,500 µg/kg	No
Mencarelli *et al*. (2013)	Lepidoptera	*Ostrinia nubilalis*	Corn	Larvae (4th instar)	14 days	Not mentioned	AFB_1	AFB_1: 125-30,000 µg/kg	*Aspergillus flavus*

Miller *et al*. (2008)	Lepidoptera	*Choristoneura fumiferana*	Spruce Trees	Larvae (2nd instar)	3 months (until 6th instar)	Not mentioned	Rugulosin	Rugulosin: 850 µg/kg (geometric mean)	No
Nevins and Grant (1971)	Diptera	*Musca domestica*	Moistened dog food pellets	Larvae (2nd instar)	7 days	Thin layer chromatography	AFB_1	AFB_1: 20 µg/kg	*Aspergillus flavusoryzae*
Niermans *et al*. (2019)	Coleoptera	*Tenebrio molitor*	Wheat flour	Larvae (42 days)	4 or 8 weeks	HPLC-MS/MS,	DON, ZEN, β-ZEL	DON: spiked with 568 and 576 µg/kg, artificially contaminated with 939 and 2,101 µg/kg and naturally contaminated with 2,854 and 4,588 µg/kg. ZEN: spiked with 589 and 2,254 µg/kg, artificially contaminated with 427 and 2,283 µg/kg and naturally contaminated with 602 and 919 µg/kg	No

Niu *et al*. (2011)	Hymenoptera	*Apis mellifera*	Bee candy (2 sucrose:1 water)	Workers	3 days	Calculations from known concentration	AFB_1, OTA	AFB_1: 500, 1,000, 2,000, 5,000, 7,000, 10,000, 15,000, 20,000 µg/kg. OTA: 1,000, 5,000, 10,000, 20,000, 40,000, 60,000 µg/kg	No
Niu *et al*. (2009)	Lepidoptera	*Amyelois transitella* and *Helicoverpa zea*	Not specified	Larvae (1st and 5th instar)	Until pupation	HPLC	AFB_1, OTA	AFB_1: 1,000, 5,000, 10,000, 20,000, 50,000 and 100,000 µg/kg. OTA: 1,000, 5,000, 10,000, 20,000 and 50,000 µg/kg	No
Niu *et al*. (2008)	Lepidoptera	*Helicoverpa zea*	Artificial diet	Larvae (5th instar)	48 hours	HPLC, , LC-MS	AFB_1	AFB_1: 1,000 µg/kg	No
Ochoa Sanabria *et al*. (2019)	Coleoptera	*Tenebrio molitor*	Wheat (naturally contaminated)	Larvae (7th-9th instar)	Until pupation	HPLC-MS	DON, 3AcDON	DON: 2,000, 10,000, 12,000 µg/kg. 3AcDON 52, 63, 205 µg/kg	No
Ohtomo *et al*. (1975)	Lepidoptera	*Bombyx mori*	Not specified	Larvae (5th instar)	5 days	Thin layer chromatography,	AFB_1	AFB_1: 15,614 µg/kg	*Aspergillus flavus* (K-199)
Patterson *et al*. (1987)	Diptera, Lepidoptera	*Drosophila melanogaster, Spodoptera littoralis*	*D. melanogaster*: maize; *S. littoralis:* beans	Larvae *(D. melanogaster:* 2nd instar and *S. littoralis:* 4th instar)	3 days	Thin layer chromatography	OTA, brevianamide A, citrinin, penicillic acid, viomellein, patulin, cyclopenol	10,000 µg/kg for all	*Penicillium*

Patterson *et al*. (1990)	Lepidoptera	*Spodoptera frugiperda* and *Heliothis virescens*	Bean based diet	Larvae (3rd-6th instar)	3 days	Preparative layer chromatography	Brevianamide A and D, OTA	Brenianamide A and B, OTA: 1,000 and 10,000 µg/kg	*Penicillium vinidicatum*
Piacenza *et al*. (2020)	Coleoptera	*Tenebrio molitor*	Oat flakes	larvae (42 days)	4 weeks	HPLC-MS/MS	T-2 and HT-2	Sum of T-2 and HT-2 (approx. 100 and 250 µg/kg)	No
Purschke *et al*. (2017)	Diptera	*Hermetia illucens*	Substrates based on corn semolina + contaminated corn grains (DON and ZEN)	Larvae (7 days)	13 days	HPLC-MS/MS QTRAP	AFB_1, AFB_2, AFG_2, OTA, DON, ZEN	DON: naturally contaminated with 4600 µg/kg, AFB_1: 88 µg/kg, AFB_2: 17 µg/kg, AFG_2: 46 µg/kg, OTA: 260 µg/kg ZEN: naturally contaminated with 860 µg/kg	No
Rizwan-UL-Haq *et al*. (2009)	Lepidoptera	*Spodoptera exigua*	Semi-synthetic diet	Larvae (neonate)	8 days	HPLC	Destruxin B	Destruxin B: 15,000, 30,000, 45,000, 60,000, 75,000, 90,000 µg/kg	*Metarhizium anisoplae*
Sadek (1996)	Lepidoptera	*Spodoptera littoralis*	Artificial diet	Larvae (2nd instar)	Whole life-cycle	Not mentioned	AFB_1, AFB_2, AFG_1	AFB_1: 500, 1,000, 2,000, 2,500, 3,000 and 3,500 µg/kg, AFB_2: 2,000, 3,000 and 4,000 µg/kg, AFG_1: 1,000, 2,000, 3,000 and 4,000 µg/kg	No

Saner *et al*. (1996)	Diptera	*Drosophila melanogaster* (strain Oregon R(R))	Not specified	-	Not applicable	Not mentioned	AFB_1	Not specified	No
Şişman *et al*. (2006)	Diptera	*Drosophila melanogaster* (strain Oregon-R)	Yeast-agar-sugar medium	Eggs, Larvae, Pupae	37-58 days	Not mentioned	AFB_1	AFB_1: 200, 500, 800 µg/kg	No
Sree and Padmaja (2008)	Lepidoptera	*Spodoptera litura*	Artificial diet	Larvae (9 days)	1, 24, 48 hours	HPLC	Destruxin	Destruxin: 88-693 µg/kg	*Metarhizium anisopliae* (M-10) and (M-19)
Sumarah *et al*. (2008)	Lepidoptera	*Choristoneura fumiferana, Lambdina fiscellaria,* and *Zeiraphera canadensis*	*Picea glauca* seedlings	Larvae (2nd and 3rd instar)	7 days	LC-MS	Rugulosin	Rugulosin: 2,713-81,375 µg/kg	*Aspergillus fumigatus*
Van Broekhoven *et al*. (2014)	Coleoptera	*Tenebrio molitor, Zophobas atratus,* and *Alphitobius diaperinus*	Maize, beer yeast, bread remains, pent grains, potato steam peelings, cookie remains	Larvae (neonate)	*T. molitor:* 26 days; *Z. atratus*: 33 days; *A. diaperinus*: 21 days	LC-MS	T-2, ZEN, OTA	T-2, ZEN, OTA: 500 µg/kg	No
Van Broekhoven *et al*. (2017)	Coleoptera	*Tenebrio molitor*	Wheat flour (spiked and naturally contaminated)	Larvae (5 weeks)	14 days	LC-MS	DON	DON: naturally contaminated with 4,900 µg/kg, spiked with: 8,000 µg/kg	No
Wright *et al*. (1976)	Coleoptera	*Tribolium confusum*	Whole wheat flour plus 5% brewer's yeast	Larvae	60 days	Not mentioned	T-2	T-2: 10,000, 100,000 µg/kg	No

Zeng *et al*. (2006)	Lepidoptera	*Helicoverpa zea*	Semi-synthetic diet containing wheat germ	Larvae (1st, 3rd and 5th instars)	13 days	Calculations from known concentration	AFB_1	AFB_1: 1, 20, 200, 1,000, 20,000 µg/kg	No
Zeng *et al*. (2013)	Lepidoptera	*Trichoplusia ni*	Semi-synthetic diet containing wheat germ	Larvae (1, 5, 7, 10 days)	6-16 days	Calculations from known concentration	AFB_1	AFB_1: 1, 20, 200, 1,000, 3,000, 5,000 µg/kg	No
Zeng *et al*. (2009)	Lepidoptera	*Helicoverpa zea*	Semi-synthetic diet containing wheat germ	Larvae (4th and 5th instar)	12 days	Calculations from known concentration	AFB_1	AFB_1: 1,000 µg/kg	No
Zhao *et al*. (2018)	Coleoptera	*Ahasverus advena*	Wheat flour, rolled oats and yeast (5:5:1 by weight)	Larvae (1, 5 ,10 days old)	4-14 days	Calculations from known concentration	AFB_1	AFB_1: 0, 500,000, 1,000,000, 2,000,000, 4,000,000, 8,000,000, and 16,000,000 µg/kg	No

Table S2
Overview of data on mortality after AFB_1 exposure for insects in the orders Diptera, Coleoptera, Lepidoptera and the species *Apis mellifera*

Species	Conc. of AFB1	Unit	Conc. shown in graph (µg/kg)	Start amount of larvae	Mortality/ Survival mentioned in study (%)	Mortality in graph (%)	Age	Exposure time	Reference
Diptera									
Drosophila melanogaster (strain A-9)	0	ppm	0	25	23.67 Adults	5	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain A-9)	0.44	ppm	440	25	0 Adults	100	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain A-9)	0.88	ppm	880	25	0 Adults	100	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain A-11)	0	ppm	0	25	22.67 Adults	9	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain A-11)	0.44	ppm	440	25	22.83 Adults	9	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain A-11)	0.88	ppm	880	25	19.67 Adults	21	Eggs	Until adult	Chinnici *et al*. (1979)
Drosophila melanogaster (strain Florida 9)	0	ppm	0	25	15.67 Adults	37	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Florida 9)	0.2	ppm	200	25	8.5 Adults	66	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Florida 9)	0.6	ppm	600	25	0.17 Adults	99	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Florida 9)	2	ppm	2,000	25	0 Adults	100	Eggs	Until adult	Gunst *et al*. (1982)

Drosophila melanogaster (strain Florida 9)	4	ppm	4,000	25	0 Adults	100	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Laussane-S)	0	ppm	0	25	17.83 Adults	29	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Laussane-S)	0.2	ppm	200	25	22.67 Adults	9	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Laussane-S)	0.6	ppm	600	25	16 Adults	36	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Laussane-S)	2	ppm	2,000	25	0.33 Adults	99	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Laussane-S)	4	ppm	4,000	25	0 Adults	100	Eggs	Until adult	Gunst *et al*. (1982)
Drosophila melanogaster (strain Oregon-R)	10	ppm	10,000	x	100 Adults	100	Eggs	8 days	Kirk *et al*. (1971)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	0	ppm	0	25	21	21	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	0.5	ppm	500	25	16	16	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	1	ppm	1,000	25	46, 54	50	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	1.3	ppm	1,300	25	82, 64, 90	79	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	1.6	ppm	1,600	25	99, 100, 95, 88	96	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	1.9	ppm	1,900	25	98, 98, 100	99	Eggs	Until adult	Melone and Chinnici (1986)

Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	2.2	ppm	2,200	25	98, 97, 97, 99	98	Eggs	Until adult	Melone and Chinnici (1986)
Drosophila melanogaster (intercrossed strain Oregon-R and Laussane-S)	2.5	ppm	2,500	25	100, 100, 100	100	Eggs	Until adult	Melone and Chinnici (1986)
Hermetia illucens	0	mg/kg ww	0	100	2	2	One-week old larvae	10 days	Camenzuli *et al*. (2018)
Hermetia illucens	0.008	mg/kg ww	8	100	0	0	One-week old larvae	10 days	Camenzuli *et al*. (2018)
Hermetia illucens	0.07	mg/kg ww	70	100	2	3	One-week old larvae	10 days	Camenzuli *et al*. (2018)
Hermetia illucens	0.39	mg/kg ww	390	100	2	2	One-week old larvae	10 days	Camenzuli *et al*. (2018)
Hermetia illucens	0	mg/kg	0	100	1	1	24 h	9 days	Meijer *et al*. (2019)
Hermetia illucens	0.5	mg/kg	500	100	3	3	24 h	9 days	Meijer *et al*. (2019)
Hermetia illucens	0	mg/kg	0	100	8	8	24 h	10 days	Bosch *et al*. (2017)
Hermetia illucens	0.1	mg/kg	100	100	9	9	24 h	10 days	Bosch *et al*. (2017)
Hermetia illucens	0.2	mg/kg	200	100	6	6	24 h	10 days	Bosch *et al*. (2017)
Hermetia illucens	0.4	mg/kg	400	100	9	9	24 h	10 days	Bosch *et al*. (2017)
Aedes aegypti	0	ppm	0	100	2	2	Freshly emerged	5 days	Matsumara and Knight (1967)
Aedes aegypti	3	ppm	3,000	100	2	2	Freshly emerged	5 days	Matsumara and Knight (1967)
Coleoptera									
Tenebrio molitor	0	mg/kg	0	100	8	8	First instar	40 days	Bosch *et al*. (2017)
Tenebrio molitor	0.1	mg/kg	100	100	3	3	First instar	40 days	Bosch *et al*. (2017)
Tenebrio molitor	0.2	mg/kg	200	100	2	2	First instar	40 days	Bosch *et al*. (2017)
Tenebrio molitor	0.4	mg/kg	400	100	3	3	First instar	40 days	Bosch *et al*. (2017)

Alphitobius diaperinus	0	mg/kg ww	0	200	26	26	Two-week old larvae	14 days	Camenzuli *et al.* (2018)
Alphitobius diaperinus	0.008	mg/kg ww	8	200	19	19	Two-week old larvae	14 days	Camenzuli *et al*. (2018)
Alphitobius diaperinus	0.07	mg/kg ww	70	200	17	17	Two-week old larvae	14 days	Camenzuli *et al*. (2018)
Alphitobius diaperinus	0.39	mg/kg ww	390	200	21	21	Two-week old larvae	14 days	Camenzuli *et al*. (2018)
Ahasverus advena	0	ppm	0	25	16	16	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	500	ppm	500,000	25	29	29	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	1,000	ppm	1,000,000	25	44	44	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	2,000	ppm	2,000,000	25	52	52	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	4,000	ppm	4,000,000	25	84	84	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	8,000	ppm	8,000,000	25	99	99	One-day old	4 days	Zhao *et al*. (2018)
Ahasverus advena	0	ppm	0	25	19	19	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	500	ppm	500,000	25	20	20	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	1,000	ppm	1,000,000	25	39	39	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	2,000	ppm	2,000,000	25	49	49	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	4,000	ppm	4,000,000	25	75	75	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	8,000	ppm	8,000,000	25	87	87	Five-days-old	9 days	Zhao *et al*. (2018)
Ahasverus advena	16,000	ppm	16,000,000	25	93	93	Five-days-old	9 days	Zhao *et al.* (2018)
Ahasverus advena	0	ppm	0	25	17	17	Ten-days-old	14 days	Zhao *et al*. (2018)

Ahasverus advena	500	ppm	500,000	25	19	19	Ten-days-old	14 days	Zhao *et al*. (2018)
Ahasverus advena	1,000	ppm	1,000,000	25	31	31	Ten-days-old	14 days	Zhao *et al*. (2018)
Ahasverus advena	2,000	ppm	2,000,000	25	33	33	Ten-days-old	14 days	Zhao *et al*. (2018)
Ahasverus advena	4,000	ppm	4,000,000	25	41	41	Ten-days-old	14 days	Zhao *et al*. (2018)
Ahasverus advena	8,000	ppm	8,000,000	25	47	47	Ten-days-old	14 days	Zhao *et al*. (2018)
Ahasverus advena	160,000	ppm	160,000,000	25	57	57	Ten-days-old	14 days	Zhao *et al*. (2018)
Lepidoptera									
Ostrinia nubilalis	2.08	log ng/g	120	15	20	20	Fourth instar	14 days	Mencarelli *et al*. (2013)
Ostrinia nubilalis	3.02	log ng/g	1,047	15	40	40	Fourth instar	14 days	Mencarelli *et al*. (2013)
Ostrinia nubilalis	4	log ng/g	10,000	15	65	65	Fourth instar	14 days	Mencarelli *et al*. (2013)
Ostrinia nubilalis	4.7	log ng/g	50,119	15	70	70	Fourth instar	14 days	Mencarelli *et al*. (2013)
Trichoplusia ni	1	µg/g	1,000	20	90	90	First instar	10 days	Zeng *et al*. (2013)
Trichoplusia ni	1	µg/g	1,000	20	100	100	First instar	16 days	Zeng *et al*. (2013)
Spodoptera littoralis	0.00	ppm	0	60	7	7	Second instar	Until pupation	Sadek (1996)
Spodoptera littoralis	0.50	ppm	500	60	13	13	Second instar	Until pupation	Sadek (1996)
Spodoptera littoralis	1.00	ppm	1,000	60	6	6	Second instar	Until pupation	Sadek (1996)
Spodoptera littoralis	2.00	ppm	2,000	60	19	19	Second instar	Until pupation	Sadek (1996)

Spodoptera littoralis	2.50	ppm	2,500	60	27	27	Second instar	Until pupation	Sadek (1996)
Spodoptera littoralis	3.00	ppm	3,000	60	33	33	Second instar	Until pupation	Sadek (1996)
Amyelois transitella	0.00	µg/g	0	20	10	10	First instar	48 h	Niu *et al*. (2009)
Amyelois transitella	1.00	µg/g	1,000	20	10	10	First instar	48 h	Niu *et al*. (2009)
Amyelois transitella	5.00	µg/g	5,000	20	15	15	First instar	48 h	Niu *et al*. (2009)
Amyelois transitella	10.00	µg/g	10,000	20	20	20	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	0.00	ng/g	0	20	2	2	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	10.00	ng/g	10	20	5	5	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	20.00	ng/g	20	20	3	3	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	40.00	ng/g	40	20	15	15	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	60.00	ng/g	60	20	50	50	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	80.00	ng/g	80	20	75	75	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	100.00	ng/g	100	20	85	85	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	120.00	ng/g	120	20	85	85	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	140.00	ng/g	140	20	90	90	First instar	48 h	Niu *et al*. (2009)
Helicoverpa zea	0.00	ng/g	0	20	20	20	First instar	15 days	Zeng *et al*. (2006)
Helicoverpa zea	1.00	ng/g	1	20	37	37	First instar	15 days	Zeng *et al*. (2006)
Helicoverpa zea	20.00	ng/g	20	20	45	45	First instar	15 days	Zeng *et al*. (2006)
Helicoverpa zea	200.00	ng/g	200	20	100	100	First instar	15 days	Zeng *et al*. (2006)
Helicoverpa zea	1.00	ug/g	1,000	20	100	100	First instar	15 days	Zeng *et al*. (2006)
Helicoverpa zea	0.00	ng/g	0	20	0	0	Third instar	21 days	Zeng *et al*. (2006)
Helicoverpa zea	20.00	ng/g	20	20	0	0	Third instar	21 days	Zeng *et al*. (2006)
Helicoverpa zea	200.00	ng/g	200	20	27	27	Third instar	21 days	Zeng *et al*. (2006)
Helicoverpa zea	1.00	µg/g	1,000	20	100	100	Third instar	21 days	Zeng *et al*. (2006)
Helicoverpa zea	20.00	µg/g	20,000	20	100	100	Third instar	21 days	Zeng *et al*. (2006)
Helicoverpa zea	0.00	ng/g	0	20	0	0	Fifth instar	10 days	Zeng *et al*. (2006)

Helicoverpa zea	20.00	ng/g	20	20	0	0	Fifth instar	10 days	Zeng *et al*. (2006)
Helicoverpa zea	200.00	ng/g	200	20	0	0	Fifth instar	10 days	Zeng *et al*. (2006)
Helicoverpa zea	1.00	µg/g	1,000	20	10	10	Fifth instar	10 days	Zeng *et al*. (2006)
Helicoverpa zea	20.00	µg/g	20,000	20	100	100	Fifth instar	10 days	Zeng *et al*. (2006)
Helicoverpa armigera	1.00	µg/g	1,000	20	40	40	Fourth instar	6 days	Elzaki *et al*. (2019)
Helicoverpa armigera	1.00	µg/g	1,000	20	55	55	Fourth instar	9 days	Elzaki *et al*. (2019)
Other									
Apis mellifera	0.00	µg/g	0	30	5	5	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	0.50	µg/g	500	30	5	5	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	1.00	µg/g	1,000	30	30	30	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	2.50	µg/g	2,500	30	20	20	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	5.00	µg/g	5,000	30	35	35	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	7.00	µg/g	7,000	30	50	50	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	10.00	µg/g	10,000	30	90	90	Newly enclosed adults	72 h	Niu *et al*. (2011)
Apis mellifera	15.00	µg/g	15,000	30	100	100	Newly enclosed adults	72 h	Niu *et al*. (2011)

Apis mellifera	20.00	µg/g	20,000	30	100	100	Newly enclosed adults	72 h	Niu *et al*. (2011)

Table S3

Concentrations of parent compounds and metabolites in initial substrate, larvae, and residual material with mass balance provided of *Hermetia illucens*, *Tenebrio molitor*, and *Alphitobius diaperinus*

Hermetia illucens

Parent compound	Type of contamination	Initial dose (µg/kg)	Initial conc. in feed (µg/kg)	Conc. in larvae (µg/kg)	Conc. in residue(s) (µg/kg)	Metabolite(s)	Initial conc. in feed (µg/kg)	Conc. in larvae (µg/kg)	Conc. in residue (µg/kg)	Total calculated mass balance %	Reference
AFB_1	S	13	0.231	< LOD	0.010	AFM_1		< LOD		4 [1]	Bosch *et al*. (2017)
	S	23	0.419	< LOD	0.032	AFM_1		< LOD		8 [1]	
	S	49	0.875	< LOD	0.047	AFM_1		< LOD		5 [1]	
	S	84	1.5	< LOD	0.110	AFM_1		< LOD		7 [1]	
	S	204	3.7	< LOD	0.275	AFM_1		< LOD		7 [1]	
	S	415	7.5	< LOD	1.3	AFM_1		< LOD		17 [1]	
AFB_1	S	13	13.3	< LOD	10.9	AFB_2	2.6	< LOD	< LOQ	48 [2]	Purschke *et al*. (2017)
						AFG_2	7	< LOD	< LOQ		
DON	S	698	697.7	< LOD	1,135.7					163 [2]	
OTA	S	39	39.4	< LOD	< LOQ					0 [2]	
ZEN	S	130	130.7	< LOD	103.7					79 [2]	
AFB_1	S	20 (ww)	8 (ww)	< LOQ	5.5 + <LOQ (dw)	AFM_1		< LOQ	< LOQ	11 [1]	Camenzuli *et al*. (2018)
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
	S	200 (ww)	70 (ww)	< LOQ	62 + 1.8 (dw)	AFM_1		< LOQ	< LOQ	18 [1]	
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
	S	500 (ww)	390 (ww)	< LOQ	303.3 + 5.3 (dw)	AFM_1		< LOQ	< LOQ	16 [1]	
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		

AFB_1 (Mix)	S	20 (ww)	18 (ww)	< LOQ	13.7 + < LOQ (dw)	AFM_1 AFL AFP_1 AFQ_1	< LOQ < LOQ < LOQ < LOQ	< LOQ < LOQ < LOQ < LOQ	16 [1]	Camenzuli *et al*. (2018)
	S	200 (ww)	180 (ww)	< LOQ	156.7 + 1.8 (dw)	AFM_1 AFL AFP_1 AFQ_1	< LOQ < LOQ < LOQ < LOQ	< LOQ < LOQ < LOQ < LOQ	17 [1]	
	S	500 (ww)	430 (ww)	< LOQ	353.3 + 4.6 (dw)	AFM_1 AFL AFP_1 AFQ_1	< LOQ < LOQ < LOQ < LOQ	< LOQ 67 + < LOQ (dw) < LOQ < LOQ	16 [1]	
DON	S	5,000 (ww)	3,900 (ww)	< LOQ	7,700 + 300 (dw)				39 [1]	
	S	50,000 (ww)	38,000 (ww)	129.3 (dw)	86,300 + 2,500 (dw)				45 [1]	
	S	125,000 (ww)	112,000 (ww)	256.7 (dw)	316,700 +5,500 (dw)				55 [1]	
DON (Mix)	S	5,000 (ww)	4,100 (ww)	< LOQ	15,700 + 200 (dw)				80 [1]	
	S	50,000 (ww)	41,000 (ww)	109.5 (dw)	150,000 + 1,800 (dw)				74 [1]	
	S	125,000 (ww)	100,000 (ww)	176.7 (dw)	296,700 + 4,200(dw)				55 [1]	

OTA	S	100 (ww)	170 (ww)	< LOQ	400 + 5.1 (dw)				53 [1]	Camenzuli *et al*. (2018)
	S	1,000 (ww)	1,300 (ww)	2.2 (dw)	3,500 +26.3 (dw)				57 [1]	
	S	2,500 (ww)	1,700 (ww)	2.6 (dw)	5,100 + 45.0 (dw)				56 [1]	
OTA (Mix)	S	100 (ww)	80 (ww)	< LOQ	200 + < LOQ (dw)				41 [1]	
	S	1,000 (ww)	800 (ww)	1.8 (dw)	2,500 + 27.3 (dw)				62 [1]	
	S	2,500 (ww)	2,000 (ww)	3.9 (dw)	5,000 + 69.0 (dw)				46 [1]	
ZEN	S	500 (ww)	280 (ww)	< LOQ	700 + < LOQ (dw)	α-ZEL	< LOQ	640 + < LOQ (dw)	104 [1]	
	S					β-ZEL	< LOQ	180 + < LOQ (dw)		
	S	5,000 (ww)	2,500 (ww)	< LOQ	6,600 +183.3 (dw)	α-ZEL	5 (dw)	7,200 + 140 (dw)	128 [1]	
	S					β-ZEL	< LOQ	2,300 + 42 (dw)		
	S	12,500 (ww)	13,000 (ww)	27.5 (dw)	35,300 + 26.3 (dw)	α-ZEL	25 (dw)	37,300 + 600 (dw)	133 [1]	
	S					β-ZEL	7 (dw)	11,200 + 180 (dw)		
ZEN (Mix)	S	500 (ww)	400 (ww)	< LOQ	990 +	α-ZEL	< LOQ	740 + 22 (dw)	100 [1]	

					< LOQ (dw)	β-ZEL		< LOQ	200 + < LOQ (dw)		
	S	5,000 (ww)	3,800 (ww)	< LOQ	10,200 + 78.7 (dw)	α-ZEL		11 (dw)	12,700 + 210 (dw)	143 [1]	Camenzuli *et al*. (2018)
						β-ZEL		< LOQ	3,600 + 71 (dw)		
	S	12,500 (ww)	9,400 (ww)	< LOQ	22,700 + 226.7 (dw)	α-ZEL		29 (dw)	28,300 + 630 (dw)	118 [1]	
						β-ZEL		6.7 (dw)	8,600 + 130 (dw)		
DON	NC, CDR		779	< LOD	1,473	3AcDON	< LOD	< LOD	< LOD	± 81 [1]	Leni *et al.* (2019)
FB_1	NC, CDR		573	< LOD	951					± 72 [1]	
FB_2	NC, CDR		441	< LOD	344					± 5 [1]	
ZEN	NC, CDR		< LOD	< LOD	334						

Tenebrio molitor

Parent compound	Type of contamination	Initial Dose (µg/kg)	Initial Conc. in Feed (µg/kg)	Conc. in Larvae (µg/kg)	Conc. in Residue (µg/kg)	Metabolite(s)	Initial Conc. in Feed (µg/kg)	Conc. in Larvae (µg/kg)	Conc. in Residue (µg/kg)	Total Calculated Mass Balance %	Reference
AFB_1	S	13	0.115	< LOD	0.005	AFM_1		< LOD		4 [1]	Bosch *et al*. (2017)
	S	23	0.209	< LOD	0.013	AFM_1		0.0017		6 [1]	
	S	49	0.437	0.001	0.035	AFM_1		< LOD		8 [1]	
	S	84	0.756	0.001	0.083	AFM_1		0.0009		11 [1]	
	S	204	1.8	0.002	0.139	AFM_1		0.0009		8 [1]	
	S	415	3.7	0.002	0.352	AFM_1		0.0011		9 [1]	
DON	NC		200	136	131	3AcDON	< LOD	66	286	234 [2]	Sanabria *et al*. (2019)
						15AcDON	< LOD	< LOD	< LOD		
						Nivalenol [3]	< LOD	< LOD	50		
	NC		2,000	127	324	3AcDON	63	66	323	31 [2]	
						15AcDON	< LOD	< LOD	< LOD		
						Nivalenol [3]	< LOD	< LOD	< LOD		
	NC		10,000	122	230	3AcDON	52	66	326	6 [2]	
						15AcDON	< LOD	< LOD	< LOD		
						Nivalenol [3]	< LOD	< LOD	51		
	NC		12,000	131	742	3AcDON	205	65	280	8 [2]	
						15AcDON	< LOD	< LOD	< LOD		
						Nivalenol [3]	< LOD	< LOD	< LOD		
DON (mix)	NC	2854	99.9	< LOD	51.6					52 [2]	Niermans *et al*. (2019)
ZEN (mix)	NC	602.3	21.1	< LOD	4.8	α-ZEL	< LOD	< LOD	1.8	48 [2]	
						β-ZEL	0.2	< LOD	3.6		
DON (mix)	NC	4588	160.6	< LOD	73.9					46 [2]	

ZEN (mix)	NC	919.3	32.2	< LOD	6.0	α-ZEL β-ZEL	< LOD 0.4	< LOD < LOD	3.8 6.2	49 [2]	
DON (mix)	AC	939	32.9	< LOD	18.5					56 [2]	
ZEN (mix)	AC	427	14.9	< LOD	6.0	α-ZEL β-ZEL	< LOD 0.4	< LOD < LOD	1.2 4.2	75 [2]	
DON (mix)	AC	2101	73.5	< LOD	39.3					53 [2]	
ZEN (mix)	AC	2283	79.9	< LOD	26.2	α-ZEL β-ZEL	< LOD 1.7	< LOD < LOD	6.8 17.3	62 [2]	Niermans *et al*. (2019)
DON (mix)	S	568	19.9	< LOD	11.5					58 [2]	
ZEN (mix)	S	589	20.6	< LOD	11.6	α-ZEL β-ZEL	< LOD < LOD	< LOD < LOD	0.3 2.0	67 [2]	
DON (mix)	S	576	20.2	< LOD	11.7					58 [2]	
ZEN (mix)	S	2254	78.9	< LOD	49.6	α-ZEL β-ZEL	< LOD 0.1	< LOD < LOD	3.8 9.0	79 [2]	
FB_1	S	50,000	44,000		31,000					38 [1]	Abado Becognee *et al*. (1997)
	S	150,000	140,000		87,000					42 [1]	
	S	450,000	398,000		276,000					39 [1]	
DON	NC	4,900		< LOD	1,140	DON3G 15AcDON		< LOD < LOD	< LOD < LOD	14 [1]	van Broekhoven *et al*. (2017)
DON	S	8,000		< LOD	4,980	DON3G 15AcDON		< LOD < LOD	< LOD < LOD	41 [1]	
T-2 (mix)	NC		88.8	< LOD	58.6	HT-2 T-2 triol T-2 tetraol	11.1	< LOD < LOD < LOD	< LOD < LOD < LOD	59 [2]	Piacenza *et al*. (2020)

T-2 (mix)	NC	262.3	< LOD	135.8	HT-2 T-2 triol T-2 tetraol	26.0	< LOD < LOD < LOD	< LOD < LOD < LOD	47 [2]	Piacenza *et al*. (2020)
T-2 (mix)	AC	53.9	< LOD	29.7	HT-2 T-2 triol T-2 tetraol	51.6	< LOD < LOD < LOD	34.1 < LOD < LOD	60 [2]	
T-2 (mix)	AC	139.8	< LOD	51.1	HT-2 T-2 triol T-2 tetraol	120.9	< LOD < LOD < LOD	34.1 < LOD < LOD	33 [2]	

Alphitobius diaperinus

Parent compound	Type of contamination	Initial Dose (µg/kg)	Initial Conc. in Feed (µg/kg)	Conc. in Larvae (µg/kg)	Conc. in Residue(s) (µg/kg)	Metabolite(s)	Initial Conc. in Feed (µg/kg)	Conc. in Larvae (µg/kg)	Conc. in Residue (µg/kg)	Total Calculated Mass Balance %	Reference
AFB_1	S	20 (ww)	8 (ww)	< LOD	5.7 + < LOQ (dw)	AFM_1		< LOQ	< LOQ	56 [1]	Camenzuli *et al*. (2018)
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
	S	200 (ww)	70 (ww)	< LOD	61.7 + <LOQ (dw)	AFM_1		< LOQ	2.0 (dw)	79 [1]	
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
	S	500 (ww)	390 (ww)	< LOD	326.7 + <LOQ (dw)	AFM_1		< LOQ	11 (dw)	69 [1]	
						AFL		< LOQ	1.5 (dw)		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
AFB_1 (Mix)	S	20 (ww)	18 (ww)	< LOD	17.7 + <LOQ (dw)	AFM_1		< LOQ	< LOQ	79 [1]	
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		
	S	200 (ww)	180 (ww)	< LOD	153.3 + <LOQ (dw)	AFM_1		< LOQ	5.5 (dw)	71 [1]	
						AFL		< LOQ	< LOQ		
						AFP_1		< LOQ	< LOQ		
						AFQ_1		< LOQ	< LOQ		

						AFM_1	< LOQ	11 (dw)		
AFB_1 (Mix)	S	500 (ww)	430 (ww)	< LOD	413.3 + <LOQ (dw)	AFL	< LOQ	1.3 (dw)	80 [1]	
						AFP_1	< LOQ	< LOQ		
						AFQ_1	< LOQ	< LOQ		
	S	5,000 (ww)	3,900 (ww)	< LOD	4,300 + <LOQ (dw)				90 [1]	
DON	S	50,000 (ww)	38,000 (ww)	< LOD	44,700 + <LOQ (dw)				96 [1]	
	S	125,000 (ww)	112,000 (ww)	< LOD	110,000 + 263.3				80 [1]	Camenzuli *et al*. (2018)
	S	5,000 (ww)	4,100 (ww)	< LOD	4,700 + <LOQ (dw)				92 [1]	
DON (Mix)	S	50,000 (ww)	41,000 (ww)	< LOD	47,300 + <LOQ (dw)				91 [1]	
	S	125,000 (ww)	100,000 (ww)	< LOD	116,700 + <LOQ (dw)				94 [1]	
	S	100 (ww)	170 (ww)	< LOD	210 + < LOQ (dw)				97 [1]	
OTA	S	1,000 (ww)	1,300 (ww)	< LOD	2,400 + <LOQ (dw)				111 [1]	
	S	2,500 (ww)	1,700 (ww)	< LOD	1,900 + <LOQ (dw)				115 [1]	

OTA (Mix)	S	100 (ww)	80 (ww)	< LOD	120 + < LOQ (dw)				116 [1]	Camenzuli *et al*. (2018)
	S	1,000 (ww)	800 (ww)	< LOD	1,100 + <LOQ (dw)				111 [1]	
	S	2,500 (ww)	2,000 (ww)	< LOD	3,100 + 2.4 (dw)				126 [1]	
ZEN	S	500 (ww)	280 (ww)	< LOD	280 + < LOQ (dw)	α-ZEL	< LOQ	23 (dw)	88 [1]	
	S					β-ZEL	< LOQ	< LOQ		
	S	5,000 (ww)	2,500 (ww)	< LOD	3,000 + <LOQ (dw)	α-ZEL	< LOQ	203 (dw)	109 [1]	
	S					β-ZEL	< LOQ	102 (dw)		
	S	12,500 (ww)	13,000 (ww)	< LOD	13,700 + <LOQ (dw)	α-ZEL	< LOQ	820 (dw)	91 [1]	
	S					β-ZEL	< LOQ	320 (dw)		
ZEN (Mix)	S	500 (ww)	400 (ww)	< LOD	490 + < LOQ (dw)	α-ZEL	< LOQ	30 (dw)	105 [1]	
	S					β-ZEL	< LOQ	11 (dw)		
	S	5,000 (ww)	3,800 (ww)	< LOD	4,300 + <LOQ (dw)	α-ZEL	< LOQ	350 (dw)	100 [1]	
	S					β-ZEL	< LOQ	140 (dw)		
	S	12,500 (ww)	9,400 (ww)	< LOD	12,700 + <LOQ (dw)	α-ZEL	< LOQ	740 (dw)	117 [1]	
	S					β-ZEL	< LOQ	270 (dw)		
DON	NC, CG		1207	726	827	3AcDON	< LOD	< LOD	< LOD	± 43 [1]	Leni *et al*. (2019)
FB_1	NC, CG		727	127	728					± 55 [1]	
FB_2	NC, CG		294	< LOD	< LOD					± 6 [1]	
DON	NC, WM		938	416	< LOD	3AcDON	< LOD	< LOD	< LOD	± 6 [1]	
DON	NC, CDR		779	468	587	3AcDON	< LOD	< LOD	< LOD	± 22 [1]	

FB_1	NC, CDR	573	< LOD	224	± 12 [1]	Leni *et al*.
FB_2	NC, CDR	441	< LOD	< LOD	± 2 [1]	(2019)

Chapter 3

Effects of the mycotoxins aflatoxin B_1, deoxynivalenol and zearalenone on survival, biomass and toxin accumulation in *Musca domestica* larvae

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Abstract

Insects are receiving increasing attention as a possible ingredient for feed and/or food production. When used efficiently, insects can provide a sustainable and economically favourable contribution to global food security. Housefly larvae (HFL) can grow on a variety of organic side streams and upgrade them by partial conversion into high-quality protein. Organic side streams may be chemically contaminated by naturally occurring toxins *e.g.* mycotoxins, therefore, effects on insect survival and biomass as well as other feed and/or food safety issues should be investigated. In this study, the HFL were exposed to a feed substrate spiked with aflatoxin B_1 (AFB_1), deoxynivalenol (DON) or zearalenone (ZEN) at concentrations of either 1 or 10 times the maximum levels (MLs) or guidance values (GVs) set for feed materials by the European Commission. Mortality and biomass of HFL were recorded over five days of exposure. LC-MS/MS analysis was used to determine the concentration of the mycotoxins in the substrate offered, the larvae and the residual feed material. A molar mass balance was calculated to estimate how much of the spiked mycotoxins (and several metabolites), was recovered in the larval body and the residual material. Exposure to either of the three mycotoxins did not affect larval mortality and biomass, and accumulation in the larval body did not take place. Metabolism does seem to occur for AFB_1 and ZEN as the molar mass balance revealed an unrecovered fraction of *ca.* 40 – 50%. Little DON metabolism occurred as most of the initially present DON was found back unchanged. The results of this study support the potential for safe use of HFL as food- and/or feed when reared on mycotoxin-contaminated side-streams, as accumulation of the tested mycotoxins did not take place in HFL. Further research is needed to identify the fate of the unrecovered fractions of AFB_1 and ZEN.

Introduction

Novel protein sources for food and feed are urgently needed, and insects are considered a valuable source of novel proteins. In order to make insect rearing circular, sustainable and economically feasible, insects could be reared on residual organic streams from agriculture or food production. Fly larvae are receiving increasing attention as they can be reared on a variety of organic residues. Such organic materials potentially contain contaminants which could accumulate in the insect body (Van der Fels-Klerx *et al.* 2018). Therefore, potential food or feed safety issues should be identified and controlled in advance. A class of contaminants commonly occurring in organic residues are mycotoxins (Van der Fels-Klerx *et al.* 2018).

Currently, mycotoxin contamination is considered as one of the most important food/feed safety challenges in the food and feed industry (Moretti *et al.* 2019). Due to climate change, a shift in the mycotoxin contamination pattern as well as geographical distribution pattern is expected (Zingales *et al*., 2022). Furthermore, modelling of different climate change scenarios predicts an expansion of risk zones leading to an increased exposure to these mycotoxins (Battilani *et al*., 2016).

Mycotoxins are secondary metabolites produced by fungi and constitute a chemically diverse class of compounds, among which several of the most potent toxic molecules of biological origin. Mycotoxins cause a variety of adverse effects in humans and animals, such as carcinogenicity, hepatoxicity, nephrotoxicity and oestrogenicity among others (Zain 2011). As mycotoxin exposure causes a variety of detrimental health effects, exposure needs to be kept below safe limits. In order to ensure this, maximum levels (ML) or guidance values (GV) for feed as well as food are set by the European Commission (EC 2002a; EC 2006b). When contamination occurs in levels above these respective levels, contaminated commodities may no longer be used as feed- and/or food. Recycling these contaminated commodities as feed substrate ('substrate' in brief) for insect larvae limits feed- and food waste and results in high quality insect proteins.

Several feeding studies on a variety of insects examined the effects of mycotoxins on insect biomass and survival as well as the possible transfer of mycotoxins from the substrate into the insect body (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Meijer *et al.* 2019; Piacenza *et al.* 2020). These studies showed that biomass and survival of the insects studied is not at all affected by the presence of mycotoxins in their substrate. A recent systematic literature review concluded that the use of mycotoxin-contaminated waste streams as substrate for insect rearing seems to provide a promising approach for the future of mycotoxin remediation and a circular economy (*Chapter 2*; Niermans *et al.* 2021). Most available studies focussed on *Hermetia illucens* L.*, Alphitobius diaperinus* Panzer and *Tenebrio molitor* L. (the latter two are beetles (Coleoptera: Tenebrionidae). The larvae of the house fly (HFL) *Musca domestica* L. (Diptera: Muscidae) are considered as an additional promising source of proteins for feed. However, data for a safety assessment for HFL fed on mycotoxin-contaminated substrates are lacking. *Hermetia illucens* and the HFL both belong to the order Diptera, but to different families and have a considerably different ecology (Kortsmit *et al.* 2022; Van Huis *et al.* 2020). Data gathered on *H. illucens* may therefore not be generally valid for Diptera.

Only few studies on HFL, dating back to the 1970s, have been published (Al-Adil *et al.* 1972; Nevins and Grant 1971). These indicate that oral uptake of aflatoxin B_1 (AFB_1) and G_1 (AFG_1) increases insect mortality, act as a temporary chemo-sterilant (Al-Adil *et al.* 1972) and resulted in a ten-fold higher concentration of AFB_1 in the HFL relative to the substrate (Nevins and Grant 1971). Studies performed on other insect species reared for food and feed, including *H. illucens*, *A. diaperinus* and *T. molitor* concluded that accumulation and transfer of mycotoxins has, until now, not been observed, although a portion of the ingested mycotoxins could not be recovered (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Charlton *et al.* 2015; Leni *et al.* 2019; Meijer *et al.* 2019; Niermans *et al.* 2019; Piacenza *et al.* 2020; Schrögel and Wätjen 2019). These unrecovered mycotoxin fractions could indicate the formation of unknown metabolites or masked forms caused by metabolic processes and interactions with other substances in the substrate (Gützkow *et al.* 2021; Meijer *et al.* 2022).

The aims of the present work were (I) to determine the effect of AFB_1, deoxynivalenol (DON) and zearalenone (ZEN) exposure on HFL survival and biomass, (II) to determine whether mycotoxin accumulation takes place in the HFL body, and (III) to get a better insight into mycotoxin metabolism by determining how much of the initially fed mycotoxins can be recovered in the larval body and the residual materials (mycotoxin recovery).

Materials and methods

Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): AFB_1, aflatoxin B_2 (AFB_2), AFG_1, aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), DON, 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON), DON-3-glucoside (DON3G); from Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and from TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

Spiked substrate preparation

Spiking solutions of AFB_1, DON and ZEN were dissolved in methanol (MeOH). Table 1 presents the intended and the final (analytically determined) concentrations in the substrate. Four mL of the spiking solution was mixed with 216 mL water, 20 mL nipagin solution in water (final concentration: 0.9 mg/L; Merck, Darmstadt, Germany) and 160 g dry food mix (37% wheat bran, 56% wheat flour, 4% full fat milk powder (28.2 g fat/100 g) and 2% dry instant baker's yeast) to obtain four separate batches of wet substrate of in total 400 mL (60% moisture; 1% MeOH). In the solvent control- and the control substrate the four mL spiking solution was either replaced by MeOH or water. The wet substrate was mixed manually for 5 min, and subsequently for 30 min in a head-over-head shaker (Reax 2, Heidolph Instruments GmbH & Co, Schwabach, Germany). The control substrates were prepared in the same way as the spiked substrates, with an equal amount of MeOH except for the blank.

In order to determine whether the spiked substrates could be considered homogeneous, 10 replicates from the AFB_1 1x ML substrate were analysed for the presence of AFB_1, and four replicates of each of the other treatment substrates were analysed. When the measured concentrations (relative standard deviation of the replicates) in the samples differed ≤ 20% from each other, the samples (and substrates) were considered homogeneous. The control substrates were also analysed in order to verify that these were free of mycotoxins.

Table 1. Overview of experimental substrates

#	Substrate	Intended mycotoxin conc. (µg/kg ww)	Actual mycotoxin conc. (µg/kg ww) [1]	Percentage of MeOH in substrate
1	Blank	0	0	0%
2	Solvent (MeOH) control	0	0	1%
3	Substrate + AFB_1 1x ML	20	22	1%
4	Substrate + AFB_1 10x ML	200	226	1%
5	Substrate + DON 1x GV	8,000	8,839	1%
6	Substrate + DON 10x GV	80,000	84,243	1%
7	Substrate + ZEN 1x GV	2,000	3,546	1%
8	Substrate + ZEN 10x GV	20,000	19,419	1%

[1] Average concentration over the replicates. Individual measurements for the replicates (n=4) can be found in Table S1. ww: wet weight; ML: maximum level; GV: guidance value.

Housefly rearing

The HF eggs used in this study were taken from the HF colony reared at the Laboratory of Entomology (Wageningen University), originally obtained from dr. Leo Beukeboom, Faculty of Science and Engineering, University of Groningen. Adult HF were kept in mesh BugDorm insect rearing cages (W24.5 x D24.5 x H24.5 cm, MegaView Science Co., Ltd.,, Taichung, Taiwan) in a climate cell with the following settings: 25°C, relative humidity of 65% and a day/night rhythm of 12/12 h. The adult flies had access to tap water, a 20% sucrose solution, full fat milk powder (28.2 g fat/100 g) and dry instant baker's yeast at all times. HF eggs were collected in a nylon sock which was hung into a paper cup above approx. five cm of full fat cow milk and covered by an aluminium lid with holes, providing an optimum environment for egg laying. The necessary number of eggs was collected from the nylon sock, and used in the current study. For colony maintenance purposes, the rest of the eggs were weighed, and placed in a 480 mL rearing cup (BugDorm insect pots purchased from MegaView Science Co., Ltd.,, Taichung, Taiwan) on top of the wet substrate (*ca.* 0.012 g of HF eggs per 62.5 g wet substrate). The wet substrate consisted of a dry food mix containing 37% wheat bran, 56% wheat flour, 4% full fat milk powder (28.2 g fat/100 g) and 2% dry instant baker's yeast. Water was added to the dry substrate mix to obtain the wet substrate (water:dry substrate mix; 60:40). Mature larvae were left in the substrate to develop into pupae and two days before the expected emergence the rearing cups were placed in new clean BugDorm insect rearing cages.

Experimental set-up

HFL were exposed to a control substrate (with or without solvent), or a substrate spiked with AFB_1, DON or ZEN. Mycotoxins were spiked to the substrate in a concentration of either 1x or 10x the ML or GV allowed by the European Commission (EC). The concentration chosen for AFB_1 was based on the ML for all feed materials, as set in Directive 2002/32/EC (EC 2002a). The chosen

concentrations for DON and ZEN were based on the lowest GV for feed materials as mentioned in EC Recommendation 2006/576/EC (EC 2006b). Table 1 presents an overview of the substrates tested. Per substrate treatment, four replicates were performed leading to a total of 32 treatment groups.

Two-hundred eggs were collected from the HFL culture and placed on top of 62.5 g mycotoxin spiked wet substrate (preparation described below) in each of the 480 mL rearing cups (BugDorm insect pots purchased from MegaView Science Co., Ltd.,, Taichung, Taiwan) and placed in a climate cell at 25°C, relative humidity of 65% and a day/night rhythm of 12/12 h. On the fifth day of exposure, larvae were separated from the residual material (a mixture of left-over substrate and frass), transferred to a clean (non-spiked) substrate for ± 4 h and collected afterwards. The residual material of the spiked substrates and the control substrates was weighed, collected and stored for further analyses. After each step, larvae were washed and their body surface dried using a paper towel. Per rearing cup both the total fresh larval biomass and the number of larvae were quantified by either weighing or counting, respectively. Larval- and residual material were stored at -20°C until further analyses.

Extraction

Before extraction, the frozen five-day-old HFL were ground under liquid nitrogen to obtain a fine powder, the sample was then stored at -80°C. Sample extraction of the substrate and residual material was performed in accordance with an in-house validated method based on the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method, with a slightly adjusted protocol for the larval samples. A quantity of 2.5 g of the substrate or residual material was weighed in a 50 mL tube, 7.5 mL water was added and shaken. After 15 min, 10 mL extraction solvent (acetonitrile, 1% acetic acid) was added and mixed for 30 min in a head-over-head shaker (Reax 2, Heidolph Instruments GmbH & Co, Schwabach, Germany). Afterwards, 4 g of magnesium sulphate was added, mixed manually and vortexed for 1 min. Samples were centrifuged (MSE Falcon 6-30) for 10 min at 3000 rpm. The same procedure was used for the HFL analyses, however, in order to prevent use of excessive amounts of larval sample material, extractions were done with 200 mg of larval sample. Amounts of solvents and sample material used in the extraction procedure were adjusted accordingly. Finally, 200 µL of sample extract was added together with 200 µL water in a syringeless PTFE filter file (Mini-UniPrep, Whatman, Marlborough, MA), capped, vortexed and placed in the refrigerator for 30 min. Afterwards, the vials were closed and stored at 4°C until LC-MS/MS analyses. In order to calculate the mycotoxin concentrations in the samples, a matrix-matched calibration was prepared in blank extract of each of the matrices (initial substrate, larvae and residual material).

Chemical analyses

The LC-MS/MS system consisted of a Waters Acquity injection and pump system (Waters, Milford, MA) and an Waters Micromass Ultima triple quad system equipped with an electrospray ionization (ESI) source which was operated in positive- and negative mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Tables S2 and S3). LC separation was performed by an Acquity HSS T3 1.8 µm 100x2.1 mm column (Waters, Milford, MA). Eluent A consisted of H_2O and eluent B was composed of MeOH:H_2O 95/5 (v/v); for the positive ionization mode both eluents contained 1 mM ammonium formate and 1% formic acid, while for the negative ionization mode both eluents contained 5 mM ammonium acetate and 0.1% acetic acid. The LC eluent gradient for both ionisation modes was similar and started with an initial period of 2 min at 100% A. The proportion of B was linearly increased to 50% at 3 min and followed by a linear gradient of 100% B at 8 min (5 min for the negative mode) and was kept for 2 min. In the positive mode, the initial conditions were restored at 10.5 min and the elution ended at 15 min, while for the negative ionisation mode this happened after 8.5 min and 11 min, respectively. The flow rate was 0.4 mL/min, the column temperature was 35°C, and the injection volume 5 µL. The conditions set for electrospray ionization (ESI) were as follows: spray voltage 2.5 kV/2.0 kV, desolvation temperature 350°C, desolvation gas flow 565 L/h. MassLynx v4.2 software (Waters, Milford, MA) was used to analyse the LC-MS/MS data obtained.

Identification of peaks was performed according to the criteria of an in-house validated method. The LOD was determined as the lowest concentration included in the calibration curve with a signal to noise ratio ≥ 3, and the LOQ was determined by following the criteria for identification and recovery mentioned in SANTE/12682/2019 (EC 2019b) and can be found in Table S4. To obtain the final mycotoxin concentration in the samples the calculated mycotoxin concentrations were corrected for the determined average recovery of the mycotoxins in the different matrices (Table S5).

All samples (substrate, larvae and residue) were analysed for the concentration of the mycotoxin (AFB_1, DON, ZEN) spiked to the substrate, and its respective metabolites (mentioned above).

Data analysis

Data on the number of larvae and larval biomass at harvest were used to determine the effect of mycotoxin exposure on HFL survival and biomass. A Kruskal-Wallis test and a Dunn's Multiple Comparison Test (comparison of all treatments with the solvent (1% MeOH) control treatment) was performed in GraphPad Prism v4 to determine whether survival and individual larval biomass measured in the treatment groups differed significantly ($P < 0.05$). A Mann-Whitney test U was performed to determine whether the absolute amount (µg) of mycotoxins differed significantly ($P < 0.05$; IBM SPSS statistics) between the substrate and residue samples. Molar mass balance calculations were performed for all substrates other than the control substrates.

Detected concentrations from the LC-MS/MS analyses were adjusted for the wet weight measured for each of the treatments. Mycotoxin concentrations below the LOQ were set to zero. The concentrations (µg/kg) of the mycotoxins determined in larval biomass and mass of residual substrate by LC-MS/MS analysis were multiplied by the total wet weight of larval biomass or residual substrate mass respectively to yield the total amount of mycotoxin recovered, including the amounts of metabolites analysed. The molar mass balance was calculated by dividing the sum of the mycotoxin mass detected in the larvae and residual material by the mycotoxin molar mass, including that of metabolites quantified, present in the initial substrate on a wet weight basis. All figures were made in GraphPad Prism v4.

Results

Control substrates and sample homogeneity

No concentrations above the LOQ for any of the three mycotoxins and their metabolites included in the analyses were detected in the control substrate. For all spiked substrates, the relative standard deviation of the mycotoxin concentration between the replicates was ≤ 20% and the mycotoxin concentrations in the substrates were therefore considered as homogeneous (Table S1).

Larval survival and biomass

The average survival of the HFL was 66 - 83% and was not affected by exposure to AFB_1, DON and ZEN for 5 days (Figure 1a). Exposure to the mycotoxins did also not result in an effect on insect biomass when comparing the individual treatments with the solvent control substrate (Figure 1b).

Mycotoxin accumulation and metabolism

Chemical analytical results showed that parent compounds and metabolites were detected in the substrate and residual materials (in concentrations above the LOQ), but that these were not present in concentrations above the LOQ in any of the larval samples.

A molar mass balance was calculated in order to express which fraction of the initially present parent compounds (or metabolites) in the substrate was recovered in the larvae and the residual material. The molar mass-balances of the AFB_1 treatments - as based on averages - were 50% (1x ML) and 63% (10x ML). Even though seven known aflatoxins (AFB_2, AFG_1, AFG_2, AFM_1, AFL, AFP_1 and AFQ_1) were analysed, neither the substrate, the larvae nor the collected residual material contained AFB_1 metabolites in concentrations above their LOQ. The absolute amount of AFB_1 (µg) in the substrate was significantly lower than in the residual material for both the 1x ML and the 10x ML substrates ($P < 0.05$).

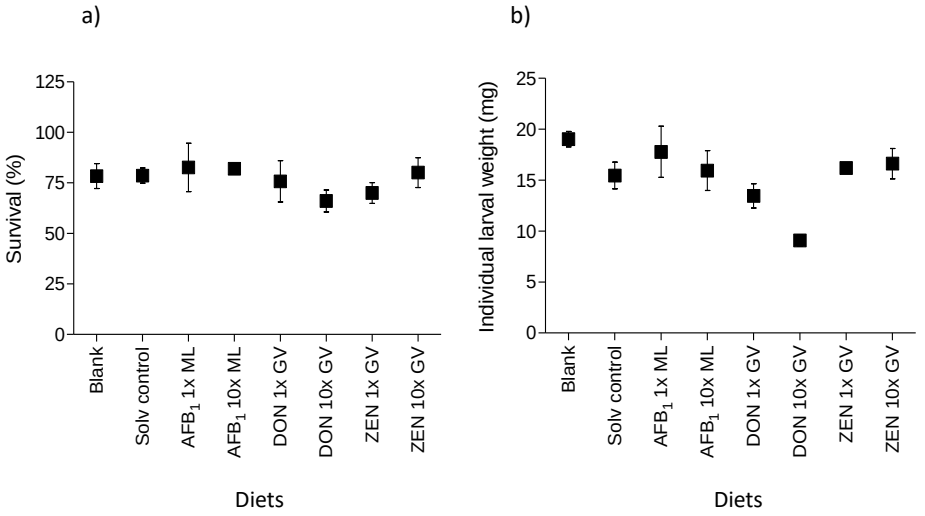

Figure 1. (a) Larval survival and (b) individual larval weight recorded after five days feeding on the included substrates. Significance was determined by comparing individual treatments with the solvent control substrate (* P = < 0.05). Error bars represent the SEM as a measure of variability between the replicates (n=4).

The calculated molar mass balance of DON in both treatments was 112% (1x GV) and 95% (10x GV) (Figure 2). This indicates that a complete mycotoxin recovery was obtained by the DON metabolites analysed. DON itself and the metabolites DON3G, 15AcDON and 3AcDON were present in the residual materials of the DON-spiked substrates. For both 1x and 10x GV DON-spiked substrates, DON was responsible for the majority of the recovered fraction, and the absolute amount of DON (µg) in the substrate did not significantly differ from the concentration in the residue in both the 1x GV (P = 0.886) and the 10x GV (P = 0.886) substrates (Table S7). Contribution of the metabolites; DON3G, 15AcDON and 3AcDON to the total amount of DON-related compounds ranged between 0.01% and 1.3%, indicating that only little metabolism occurred (Table S6).

The molar mass balance of ZEN was 54% (1x GV) and 63% (10x GV) (Figure 2). In the mass balance of both ZEN treatments, ZEN was the main compound recovered. α- and β-ZEL contributed – on average - 4.1% and 4.7% to the mass balance of the ZEN 1x GV substrate. In the ZEN 10x GV substrate, α- and β-ZEL contributed around 2.3% and 8.3% to the mass balance, respectively (Table S6). As mentioned above, no metabolites were detected in the substrate and residual material of the AFB_1 treatments and for DON no significant difference in absolute concentration between the substrate and residual material was found (Table S7).

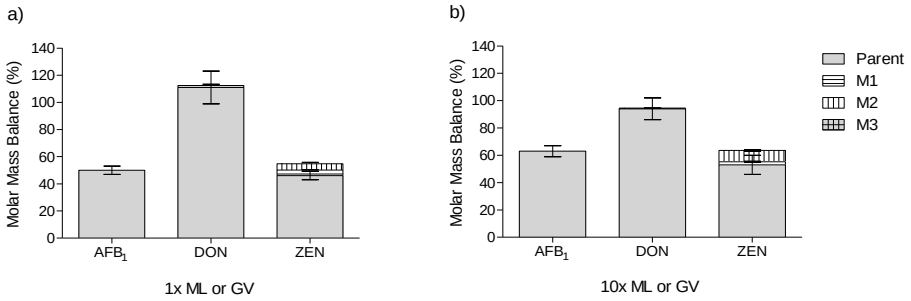

Figure 2. Molar mass balance of AFB_1, DON and ZEN for each of the spiked substrates: a) maximum level (ML) or guidance value (GV) and b) 10x maximum level (ML) or guidance value (GV). Error bars represent the SEM as a measure of variability between the replicates (n=4). Metabolites (M) contributing to the mass balance for DON were M1: DON3G, M2: 15AcDON, and M3: 3AcDON and for ZEN were M1: α-ZEL and M2: β-ZEL. None of the AFB_1 metabolites were detected in any of the samples in quantifiable concentrations. A table with the average contribution of the four replicates and the SEM per parent compound or metabolite can be found in Appendix F.

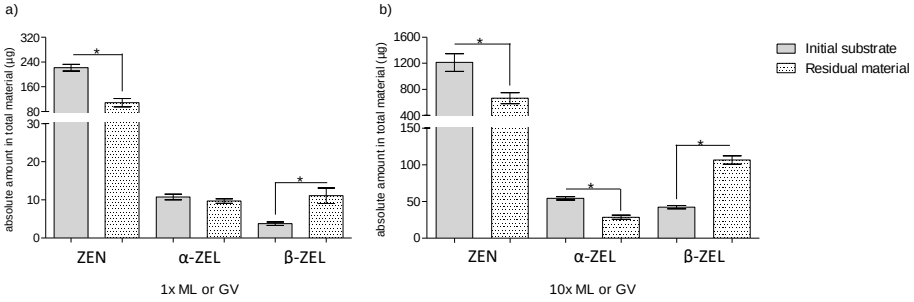

Figure 3. Overview of the absolute amount of ZEN, α- and β-ZEL (µg per kg total material) in the substrate, larvae and residues for each of the two substrates: a) ZEN 1x GV and b) ZEN 10x GV. Bars indicate the absolute total amount (µg) of mycotoxin (metabolite) recovered in the initial substrate after spiking or in the residual material. Significance was determined by comparing the concentration of the respective toxin in the substrate with the concentration of the same toxin in the residual material belonging to the same substrate (* = $P < 0.05$). Error bars represent the SEM as a measure of variability between the replicates (n=4), an overview per replicate can be found in Appendix G.

In the case of ZEN and its metabolites the absolute amount and their contribution – though low - in the substrate and residual material was calculated (Figures 3a and 3b). The metabolites α- and β-ZEL were already present in the ZEN-spiked substrates. Interestingly, the concentration of ZEN is significantly ($P < 0.05$) decreased in the residual material of both the 1x and 10x GV ZEN-spiked substrates as compared to the initial substrate (Figures 3a and b). Also the absolute concentration (µg) of α-ZEL, in the 10x GV substrate, and β-ZEL, in both ZEN substrates, changed significantly ($P < 0.05$).

Discussion

Our study is the first to evaluate the effects of mycotoxin exposure of HFL using LC-MS/MS and to present molar mass balances quantifying compound recovery. Survival of HFL was not significantly affected by mycotoxin exposure. Exposure started in the egg stage and consequently survival reflects effects on hatchability and exposure of neonates to the mycotoxins. Furthermore, the presence of unfertilized eggs (Gadallah and Marei 1973) or damage caused while handling the eggs could have contributed to the mortality that occurred in this study. No other comparable studies on HFL have been performed. Studies on *H. illucens* larvae (also order Diptera, class Brachycera, but family Stratiomyidae instead of Muscidae) did not start in the egg phase, but in a later larval stage (five or seven days since hatching, when the larvae were switched from a starter substrate to the organic residue substrate) and therefore likely resulted in a higher survival (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Meijer *et al.* 2019). HFL develop into pupae already on day six or seven and no switch from starter to organic residue substrate is applied in practice. We therefore started exposure in the egg stage to match the practice of commercial HFL mass rearing. A study on *Drosophila melanogaster* Meigen strain A11 showed that eggs exposed to AFB_1 were less likely to develop into adults than when two-day-old larvae were exposed to the same concentration of AFB_1 (Chinnici *et al.* 1979). No significant effect on HFL body mass gain was found when fed mycotoxins in concentrations corresponding to 1x and 10x the ML or GV of AFB_1, DON and ZEN. Studies on *H. illucens* showed that exposure of larvae to concentrations of AFB_1, DON and ZEN in lower (Purschke *et al.* 2017) and in even higher concentrations (Camenzuli *et al.* 2018; Meijer *et al.* 2019) than tested in this study also showed no effect on body mass.

None of the mycotoxins and metabolites included in the analyses were detected in the larval samples and therefore accumulation in the larvae did not take place. This is in contrast with a previous study in which a ten-fold higher concentration of AFB_1 in 2nd instar HF larvae was found after two days of exposure to 20 µg/kg AFB_1 (Nevins and Grant 1971). The results from the present study are in accordance with previous studies on *H. illucens*, *A. diaperinus* and *T. molitor*, showing that AFB_1, DON and ZEN did not accumulate in the insect body (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Niermans *et al.* 2019).

The results of this study lead us to infer that mycotoxin metabolism occurs in HFL. Additionally, substrate-specific enzymes and microorganisms or photochemical degradation over time could be responsible for mycotoxin metabolism or breakdown leading to an incomplete mass balance. These events were not examined in the current study and therefore no statements can be made about this.

For AFB_1 *ca.* 40 – 50% is not accounted for in the molar mass balance, even though seven aflatoxins were included in the analyses. This finding suggests that as yet unknown metabolites

are formed. It is important to trace detoxification pathways and resulting metabolites for AFB_1 in view of the future use of HFL larvae as feed/food. The percentage of AFB_1 recovered in this study seems comparable to what was found for *A. diaperinus*, while AFB_1 recovery in *H. illucens* seems lower (Camenzuli *et al.* 2018). This suggests that aflatoxin metabolism and the possible formation of unknown metabolites might also be relevant for these species. Though no aflatoxin metabolites were detected in the residual materials of the HFL, some residual materials of *A. diaperinus* contained AFL and AFM_1, and AFL was also detected in residual materials of *H. illucens* (Camenzuli *et al.* 2018). The formation of novel AFB_1 metabolites was confirmed for *T. molitor* (Gützkow *et al.* 2021). This suggests differences in metabolism routes between insect species. However, these insect species were reared on distinct substrates which might have been responsible for metabolite formation. Other studies observed low aflatoxin recovery rates after spiking a food/feed matrix (Sulyok *et al*., 2020; Warth *et al*., 2012). This suggests that non-enzymatic conjugation of AFB_1 to, for example, proteins in the matrix could be an additional a reason for the incomplete mass balance.

The molar mass balance of DON showed that DON was completely recovered in this study. Most of the DON was found back unchanged, however, DON3G, 3AcDON and 15AcDON were found in low concentrations in the residual material. These three DON metabolites seem to be the main metabolites of DON formed. Also for DON, the percentage recovered in this study was comparable to what was found for *A. diaperinus*, but it was lower in *H. illucens* (Camenzuli *et al.* 2018). The residual materials of *A. diaperinus* and *H. illucens* were also analysed for the presence of 15AcDON and DON3G, but neither of these metabolites were detected (Camenzuli *et al.* 2018), suggesting a difference in metabolism routes occurs in a HFL and BSFL study.

The molar mass balance for ZEN varied between 54% and 63%, depending on the initial concentration, indicating that 37 – 46% had been converted. Although the metabolites α- and β-ZEL were already present in the ZEN-spiked substrates, changes in their concentrations were observed, indicating a possible metabolism of ZEN into metabolites. The concentration of ZEN decreased in the residual material as compared to the concentration in the initial substrate. The concentration of β-ZEL in the residue was significantly higher in both ZEN substrates as compared to that in the spiked substrate. A preferred conversion of ZEN into β-ZEL rather than into α-ZEL has also been observed in bovine liver preparations and liver subfractions from laying hens, but seemed to depend on the species as in pigs mostly α-ZEL is formed (EFSA 2011a; EFSA 2017b). The relative potency factor (RPF) for oestrogenicity determined for α-ZEL is 60.0, (RPF ZEN is 1.0), while the RPF for β-ZEL is 0.2 (EFSA 2016). Therefore, it seems promising that HFL may convert some of the more toxic ZEN into the less toxic metabolite β-ZEL.

ZEN recovery in HFL was similar to that observed in *T. molitor* (Niermans *et al.* 2019), while ZEN recovery in *H. illucens* and *A. diaperinus* seemed to be complete (Camenzuli *et al.* 2018). Also in these insects species, α- and β-ZEL were, as in this study, present in the residual materials

(Camenzuli *et al.* 2018; Niermans *et al.* 2019). α-ZEL even contributed to around 50% of the total ZEN recovery in *H. illucens* (Camenzuli *et al.* 2018). Further studies are needed to clarify the contribution of the HFL and the substrate in this.

In conclusion, data collected in this study contribute to understanding whether HFL reared on mycotoxin-contaminated side streams could be used safely as feed and- or food ingredients. This study suggests that mycotoxin metabolism occurs in HFL, as for AFB_1 and ZEN, which were two of the three investigated mycotoxins, recovery of the initial amount of mycotoxin present in the substrates was partial (*ca*. 50 – 60%). Most of the initially present DON was found back unchanged, suggesting that little DON metabolism occurred. Therefore, further studies are needed to examine the role of the HFL as well as its substrate in mycotoxin metabolism.

Contamination with unknown metabolites can lead to an underestimation of toxicity and exposure. Therefore, further research is needed to identify the currently missing fraction of AFB_1 and ZEN. For future research it would also be of interest to include other known mammalian phase I metabolites as zearalanone (ZAN) and phase II glucosides, glutathione conjugates and sulphate esters commonly found in insects (Wilkinson 1986). Additionally, analysis of conjugates such as sulphates or glucosides which are known to be formed by plant enzymes (Brodehl *et al.* 2014), might help in determining the role of the substrate in mycotoxin metabolism.

Tracking of conversion or metabolic pathways through *e.g.* isotopic labelling are required to determine the presence of unknown metabolites to map AFB_1 metabolism in HFL. Such metabolites do not seem to be toxic to HFL, however, they might confer toxicity to consumers of HFL. Overall this study showed that HFL mortality and biomass were not affected after exposure to AFB_1, DON and ZEN. Furthermore, HFL do not accumulate the tested mycotoxins indicating their possible safe use as food- and/or feed when reared on mycotoxin-contaminated side-streams. Mycotoxin metabolism seems to occur for AFB_1 and ZEN, while metabolism of DON seems limited.

Acknowledgements

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Supplementary material

Table S1
Concentration (µg/kg) of the mycotoxin measured in the different replicates of the initial substrates

Treatments/ Replicates	AFB_1 (µg/kg)		DON (µg/kg)		ZEN (µg/kg)	
	1x ML	10x ML	1x GV	10x GV	1x GV	10x GV
1	23.8	294.6	7,486.4	76,205.9	3,127.3	25,567.0
2	23.5	190.5	8,096.5	63,650.5	3,415.1	15,378.6
3	24.3	218.8	8,679.1	99,142.3	3,699.4	18,401.2
4	20.1	198.2	11,093.7	97,973.3	3,940.9	18,328.7
5	17.7					
6	25.0					
7	20.4					
8	20.7					
9	20.9					
10	18.7					
Average	21.5	225.6	8,838.9	84,243.0	3,545.7	19,418.9
SD	2.4	41.2	1,368.4	14,993.0	304.9	3,753.2
RSD	11%	18%	15%	18%	9%	19%

Table S2
Instrumental MS/MS parameters of mycotoxins analysed in positive ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	Cone Voltage (V)	CE (V)
15AcDON (ql)	3.68	355.9	321.1	25	11.0
15AcDON (qn)	3.68	355.9	137.1	25	15.0
AFB_1 (ql)	4.79	313.0	128.0	25	61.0
AFB_1 (qn)	4.79	313.0	285.0	25	22.0
AFB_1 (ql2)	4.96	313.1	241.0	25	36.0
AFB_2 (ql)	4.70	315.1	259.2	25	29.0
AFB_2 (qn)	4.70	315.1	287.2	25	25.0
AFG_1 (ql)	4.36	329.0	200.0	25	35.0
AFG_1 (qn)	4.36	329.0	243.2	25	26.0
AFG_2 (ql)	4.07	331.1	245.2	25	29.0
AFG_2 (qn)	4.07	331.1	313.2	25	23.0
AFP_1 (qn)	4.46	299.0	271.0	25	22.0
AFP_1 (ql1)	4.46	299.0	114.9	25	47.0
AFP_1 (ql2)	4.46	299.0	90.9	25	45.0
AFQ_1 (qn)	3.94	329,1	310.8	25	19.0
AFQ_1 (ql1)	3.94	329,1	177.0	25	30.0
AFQ_1 (ql2)	3.94	329.1	128.0	25	45.0
AFL (qn)	5.60	297.1	269.0	25	19.0
AFL (ql1)	5.60	297.1	114.9	25	45.0
AFL (ql2)	5.60	297.1	141.0	25	43.0
AFM_1 (qn)	3.94	328.9	272.9	25	22.0
AFM_1 (ql)	3.94	328.9	229.0	25	37.0
DON (ql)	2.98	297.0	231.0	25	11.0
DON (qn)	2.98	297.0	249.0	25	10.0

qn: quantifier ions. ql: qualifier ions

Table S3
Instrumental MS/MS parameters of mycotoxins analysed in negative ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	Cone voltage (V)	CE (V)
3AcDON (ql)	4.55	397.1	59.0	25	35.0
3AcDON (qn)	4.55	397.1	337.0	25	8.0
DON3G (ql)	3.43	517.2	247.0	25	21.0
DON3G (qn)	3.43	517.2	457.1	25	12.0
β-ZEL (ql)	6.20	319.3	130.0	25	31.0
β-ZEL (qn)	6.20	319.3	160.0	25	27.0
ZEN (ql)	6.46	317.1	131.1	25	24.0
ZEN (qn)	6.46	317.1	175.0	25	21.0
α-ZEL (ql)	6.39	319.3	130.0	25	31.0
α-ZEL (qn)	6.39	319.3	160.0	25	27.0

qn: quantifier ions. ql: qualifier ions

Table S4
LOD and LOQ (ng/mL) per mycotoxin in the different matrices

Compound		AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1
Substrate	LOD	0.1	0.1	0.05	0.05	1	0.1	0.25	0.25
	LOQ	0.2	0.1	0.1	0.25	1	0.5	0.5	0.5
Larvae	LOD	0.1	0.01	0.05	0.05	0.25	0.25	0.5	0.1
	LOQ	0.1	0.25	0.1	0.1	1	0.25	0.5	0.5
Residue	LOD	0.2	0.	0.1	0.25	12.5	1	2.5	0.5
	LOQ	0.2	0.25	0.5	0.5	12.5	12.5	12.5	12.5

Compound		DON	DON3G	15AcDON	3AcDON	ZEN	α-ZEL	β-ZEL
Substrate	LOD	40	4	4	10	10	0.34	0.3
	LOQ	40	20	10	20	10	1.69	7.5
Larvae	LOD	40	10	2	4	10	1.69	0.75
	LOQ	40	200	4	100	10	1.69	3
Residue	LOD	40	10	10	100	10	1.69	1.5
	LOQ	40	40	40	100	10	16.94	37.5

Table S5
Recovery per mycotoxin (based on QC samples) in different matrices

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1
Substrate	75%	99%	89%	89%	73%	109%	130%	119%
Larvae	86%	82%	79%	99%	70%	117%	71%	43%
Residue	70%	98%	95%	84%	76%	127%	70%	61%

Compound	DON	DON3G	15AcDON	3AcDON	ZEN	α-ZEL	β-ZEL
Substrate	70%	80%	65%	83%	68%	64%	60%
Larvae	98%	84%	60%	122%	124%	79%	125%
Residue	71%	138%	85%	138%	158%	86%	72%

Table S6
Overview of the average contribution of AFB_1, DON and ZEN and their metabolites to the overall molar mass balance (%)

Substrates	Parent compound		M1		M2		M3		M4		M5		M6		M7		Total	
	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM
AFB_1 1x ML	50.0	0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	50.0	0.03
AFB_1 10x ML	63.0	0.04	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	63.0	0.04
DON 1x GV	111.0	0.12	1.32	0.011	0.2	0.002	nd	0.00									112.0	0.12
DON 10x GV	94.0	0.08	0.36	0.001	0.01	0.0001	0.02	0.0002									95.0	0.08
ZEN 1x GV	46.0	0.03	4.11	0.001	4.7	0.007											54.0	0.03
ZEN 10x GV	53.0	0.07	2.29	0.004	8.3	0.005											63.0	0.08

Metabolites (M1 – M7) analysed for AFB_1 were: M1: AFB_2; M2: AFG_1; M3: AFG_2; M4: AFM_1; M5: AFL; M6: AFP_1 and M7: AFQ_1. Metabolites contributing to the mass-balance for DON were M1: DON3G, M2: 15AcDON, and M3: 3AcDON and for ZEN were M1: α-ZEL and M2: β-ZEL. nd: none of the seven AFB_1 metabolites and 3AcDON were detected in any of the samples in quantifiable concentrations. This table shows the average contribution of the four replicates, an overview per replicate can be found in Table S7.

Table S7

Overview of the absolute amount of mycotoxin (metabolite) recovered (µg) in the total substrate, larvae and residues calculated per total kilogram of material

	Substrate				Larvae			Residual material				
Substrates	Parent	M1	M2	SUM	Parent	M1	M2	Parent	M1	M2	M3	SUM
AFB_1 1x ML (1)	1.5	< LOQ	< LOQ	1.5	< LOQ	< LOQ	< LOQ	0.6	< LOQ	< LOQ	< LOQ	0.6
AFB_1 1x ML (2)	1.5	< LOQ	< LOQ	1.5	< LOQ	< LOQ	< LOQ	0.8	< LOQ	< LOQ	< LOQ	0.8
AFB_1 1x ML (3)	1.5	< LOQ	< LOQ	1.5	< LOQ	< LOQ	< LOQ	0.7	< LOQ	< LOQ	< LOQ	0.7
AFB_1 1x ML (4)	1.3	< LOQ	< LOQ	1.3	< LOQ	< LOQ	< LOQ	0.7	< LOQ	< LOQ	< LOQ	0.7
AFB_1 10x ML (1)	18.4	< LOQ	< LOQ	18.4	< LOQ	< LOQ	< LOQ	9.4	< LOQ	< LOQ	< LOQ	9.4
AFB_1 10x ML (2)	11.9	< LOQ	< LOQ	11.2	< LOQ	< LOQ	< LOQ	8.2	< LOQ	< LOQ	< LOQ	8.2
AFB_1 10x ML (3)	13.7	< LOQ	< LOQ	13.7	< LOQ	< LOQ	< LOQ	8.2	< LOQ	< LOQ	< LOQ	8.2
AFB_1 10x ML (4)	12.4	< LOQ	< LOQ	12.4	< LOQ	< LOQ	< LOQ	8.7	< LOQ	< LOQ	< LOQ	8.7
DON 1x GV (1)	467.9	9.0	< LOQ	476.5	< LOQ	< LOQ	< LOQ	675.7	< LOQ	< LOQ	< LOQ	675.7
DON 1x GV (2)	506.0	< LOQ	< LOQ	506.0	< LOQ	< LOQ	< LOQ	641.4	< LOQ	3.5	< LOQ	644.9
DON 1x GV (3)	542.4	< LOQ	< LOQ	542.5	< LOQ	< LOQ	< LOQ	450.4	28.6	< LOQ	< LOQ	479.0
DON 1x GV (4)	693.4	2.0	< LOQ	695.4	< LOQ	< LOQ	< LOQ	634.3	< LOQ	< LOQ	< LOQ	634.3
DON 10x GV (1)	4762.9	65.0	< LOQ	4828.1	< LOQ	< LOQ	< LOQ	5282.7	15.5	< LOQ	< LOQ	5298.2
DON 10x GV (2)	3978.2	85.0	< LOQ	4062.8	< LOQ	< LOQ	< LOQ	4735.1	9.7	< LOQ	< LOQ	4744.8
DON 10x GV (3)	6196.4	94.0	1.0	6291.3	< LOQ	< LOQ	< LOQ	5019.5	18.6	1.7	5.1	5045.0
DON 10x GV (4)	6123.3	80.0	< LOQ	6203.5	< LOQ	< LOQ	< LOQ	5981.1	42.1	< LOQ	< LOQ	6023.2
ZEN 1x GV (1)	195.5	12.0	3.0	210.9	< LOQ	< LOQ	< LOQ	77.6	9.2	11.7		98.5
ZEN 1x GV (2)	213.4	9.0	3.0	225.7	< LOQ	< LOQ	< LOQ	95.4	8.3	5.2		108.8
ZEN 1x GV (3)	231.2	12.0	4.0	246.9	< LOQ	< LOQ	< LOQ	128.5	10.8	13.4		152.7
ZEN 1x GV (4)	246.3	10.0	5.0	261.0	< LOQ	< LOQ	< LOQ	133.7	10.5	14.1		158.4
ZEN 10x GV (1)	1597.9	59.0	48.0	1705.7	< LOQ	< LOQ	< LOQ	508.3	24.1	123.4		655.8
ZEN 10x GV (2)	961.2	48.0	38.0	1046.2	< LOQ	< LOQ	< LOQ	527.3	36.4	101.8		665.5
ZEN 10x GV (3)	1150.1	55.0	44.0	1248.7	< LOQ	< LOQ	< LOQ	803.0	24.4	103.0		930.4
ZEN 10x GV (4)	1145.5	55.0	39.0	1239.4	< LOQ	< LOQ	< LOQ	819.9	28.9	97.4		946.2

None of the seven included AFB_1 metabolites were found in levels above the LOQ in the substrate, larvae and residual materials. Metabolites contributing to the mass-balance for DON were M1: DON3G, M2: 15AcDON, and M3: 3AcDON and for ZEN were M1: α-ZEL and M2: β-ZEL. Colours are added to improve readability. For the metabolites which are not mentioned in the table or not filled in the concentration found was < LOQ.

Chapter 4

The metabolic fate and biological effects of isotopically labelled aflatoxin B_1, fumonisin B_1, ochratoxin A and zearalenone in reared *Hermetia illucens* and *Musca domestica* larvae

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Submitted

Abstract

Larvae of the black soldier fly (BSFL) and the house fly (HFL) are able to feed on a variety of organic side streams otherwise considered unsuitable as feed and food. In previous feeding experiments with mycotoxin-contaminated feed substrates the molar mass balance was mostly incomplete, which indicated the formation of unknown metabolites. Therefore, in the current study, BSFL and HFL were exposed to substrates spiked with regular and isotopically labelled aflatoxin B_1, fumonisin B_1, ochratoxin A or zearalenone. The molar mass balance was found complete for ochratoxin A (BSFL, HFL) and zearalenone (BSFL). For all tested mycotoxins insect survival and biomass were not affected and bio-accumulation in the insects did not occur. No isotopic labels were found back in unknown compounds except for two possibly new zearalenone metabolites detected in both insect species. Overall, mass balances for aflatoxin B_1 and zearalenone (HFL) remained incomplete, indicating the need for further research.

Introduction

Mycotoxins are secondary metabolites produced by toxigenic moulds. Large amounts of food and feed materials are contaminated by mycotoxins worldwide, having a substantial socio-economic impact (Madala *et al.* 2019). In 1999, the FAO stated that at least 25% of the food crops, worldwide, were contaminated by mycotoxins (Park *et al.* 1999). However, a recent study indicated that this was a great underestimation and stated that the prevalence of detected mycotoxins in food crops is 60-80% (Eskola *et al.* 2020). Mycotoxins, such as aflatoxins, ergot alkaloids, fumonisins, ochratoxins, trichothecenes and zearalenone, can lead to adverse effects on animal and human health via mycotoxin exposure related to feed or food consumption (Benkerroum 2020; Marin *et al.* 2013; Zain 2011). In addition to economic losses due to disease burden caused by mycotoxins, crop farmers are affected directly by reduced revenues for their production (Agriopoulou *et al.* 2020), while livestock farmers are economically affected due to reduced animal performance (Zain 2011).

In the past years, the concept of organic waste management using insects (Gold *et al.* 2018) and insect-based bioconversion has received increased attention (Van Huis 2016; Van Huis 2020). Certain insect species are able to feed and thrive on a variety of side streams or by-products that may otherwise be unsafe for conventional livestock species. If the safety of rearing insects on mycotoxin-contaminated streams could be proven, insect rearing could become a sustainable practice and contribute to circularity in the production of food and/or feed (Van der Fels-Klerx *et al.* 2018). A recent literature review concluded that the use of mycotoxin-contaminated waste streams as feed substrate ('substrate' in brief) for insect rearing has potential to provide an alternative for the future of mycotoxin remediation and to contribute to a circular economy (*Chapter 2*; Niermans *et al.* 2021). Most studies including insects focussed on aflatoxin B_1 (AFB_1), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN). Safety assessments on black

soldier fly larvae (BSFL; *Hermetia illucens* L.) (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Meijer *et al.* 2019) and housefly larvae (HFL; *Musca domestica* L.) (*Chapter 3*; Niermans *et al.* 2023a) showed that mycotoxin metabolism occurs, but that in most cases a full recovery of the initial amount of the mycotoxin present in the substrate was not achieved. This missing fraction could possibly be explained by the presence of modified mycotoxins or unknown metabolites, which can lead to an underestimation of larval toxicity and animal/human exposure. Therefore, further research is needed to track the fate of the missing fractions of AFB_1, OTA and ZEN, and novel analytical methods will need to be used. In the studies cited above, the presence of mycotoxin (metabolites) was determined by a targeted LC-MS/MS based method. Therefore, in the current study, BSFL and HFL were exposed to a substrate spiked with regular- and isotopically (^{13}C) labelled AFB_1, FB_1, OTA or ZEN. After exposure, the substrate, larval- and residual materials were collected and analysed by LC-MS/MS, and additionally tracking of the isotopic label by high resolution mass spectrometry (HRMS) was performed. The aims of the present work were (I) to determine the effect of mycotoxin exposure on BSFL and HFL survival and growth, (II) to determine whether mycotoxin accumulation takes place in the BSFL and HFL body, and (III) to track metabolic conversion of the mycotoxins.

Materials and methods

Experimental set-up

BSFL and HFL were exposed to substrates spiked with either isotopically labelled ('labelled' in brief) and regular AFB_1, FB_1, OTA or ZEN, a non-spiked control substrate or a non-spiked control substrate with solvent (MeOH). When tracking metabolic conversion of the included mycotoxins, the use of labelled mycotoxins provides additional confidence that the detected signals with a correct labelling status are metabolites of the mycotoxins. Whereas AFB_1, OTA and ZEN have been investigated before, fumonisin B_1 (FB_1) was also included in this study because it is a commonly occurring trichothecene found in food and it is regulated by the European Commission (EC) (Alshannaq and Yu 2017; EC 2006c; Marin *et al.* 2013). For DON, a complete mass balance was obtained in a previous HF experiment (*Chapter 3*; Niermans *et al.* 2023a) and it was, therefore, excluded from the current study. The concentration chosen for AFB_1 was based on the highest of the maximum limit (ML) values listed for all feed materials mentioned in Directive 2002/32/EC (EC 2002a). For OTA and ZEN, the intended spiked concentrations were based on the highest guidance values (GV) for complementary and complete feed stuffs as mentioned in EC Recommendation 2006/576/EC. For FB_1, 0.1 of the lowest GV was chosen (EC 2006b), because no data of FB_1 toxicity on the selected insect species are available. Table 1 presents an overview of the treatments included. Each of the eight substrate treatments and the two control substrate treatments were performed in triplicate.

*Table 1***.** Intended and actual analysed concentrations (µg/kg) of spiked and control substrates for the BSFL and HFL.

Substrates	Intended concentration	BSFL Corrected analysed concentration	HFL Corrected analysed concentration
Substrate Control	0	< LOD	< LOD
Solvent Control	0	< LOD	< LOD
AFB_1	20	22.0 (± 1.3)[a]	27.0 (± 1.3)[a]
$^{13}C_{17}$-AFB_1	20	22.0	22.9
FB_1	500	533	599
$^{13}C_{34}$-FB_1	500	417	426
OTA	100	95.4	94.0
$^{13}C_{20}$-OTA	100	91.4	91.5
ZEN	500	497	641
$^{13}C_{18}$-ZEN	500	558	571

[a]± standard deviation; only provided for the AFB_1 spiked substrates, as these were analysed to confirm homogeneity. The other concentrations shown were based on the analyses of one sample.

Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): [$^{13}C_{17}$]-AFB_1, AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), [$^{13}C_{34}$]-FB_1, FB_1, [$^{13}C_{20}$]-OTA, OTA, ochratoxin B (OTB), ochratoxin-alpha (OTα), [$^{13}C_{18}$]-ZEN, ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL); Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

Spiked substrate preparation

Spiking solutions of both the labelled and regular AFB_1, OTA and ZEN were prepared in methanol (MeOH) and in MeOH/H_2O (50:50 v/v) for FB_1.

BSFL substrate consisted of 35% wheat bran (Meelfabriek de Jongh, Steenbergen, the Netherlands) and 65% tap water. Prior to spiking, the wheat was milled to a particle size of 1 mm (Retsch Grindomix GM 200). The batches for the spiked substrates were made with 31.5 g (for substrates containing labelled mycotoxins) and 35 g (for regular mycotoxins) substrate, with intended concentrations as shown in Table 1. In order to create a homogenous slurry, the prepared spiking solution together with approximately 75 or 80 mL methanol was added to the spiked substrate. Each batch of slurry was mixed using a 300 W hand mixer (Philips, Eindhoven, the Netherlands) until being homogeneous and subsequently transferred to an open aluminium container. These containers were placed overnight in a fume hood for the methanol to evaporate, until the weight change was negligible. The next day, the dried batches of substrate were distributed into three replicate containers (SPL Life Sciences Co., Ltd., Gyeonggi-do, South

Korea). Each replicate container contained 8.75 ± 0.05 g of dried substrate and approximately 16.25 mL water.

In order to prepare the HF substrate, the spiking solution was mixed with water, nipagin solution (final concentration: 0.9 mg/L; Merck, Darmstadt, Germany) and a dry substrate mix containing 37% wheat bran, 56% wheat flour, 4% full fat milk powder (28.2 g fat/100 g) and 2% dry instant baker's yeast. The volume of prepared spiked substrate was such to obtain ten separate batches of wet substrate of in total 105 g (labelled) and 130 g (regular) (60% moisture; 0.5% MeOH). The wet substrate was mixed manually for 5 min and 30 min in a head-over-head shaker (Reax 2, Heidolph Instruments GmbH & Co, Schwabach, Germany).

For both insect species, the two control substrates were prepared in the same way as the spiked substrates. However, the control substrates did not contain MeOH while the percentage of MeOH in the solvent control substrate was the same as in the spiked substrates. The spiking method used in the current study was the same as in Meijer et al. (2022), and was tested for conformity with ISO standard 13528:2015 (Meijer *et al.* 2022). As the same spiking method was used in the current study six (BSF) or ten (HF) aliquots from the regular AFB_1 substrate were analysed for the presence of AFB_1 to confirm homogeneity. When the relative standard deviation of the replicates of the analysed samples of one treatment was ≤ 20%, the substrate was considered homogeneous. The control substrates were also analysed for the presence of all mycotoxins and metabolites considered in this study (to verify whether these were absent). One sample of each of the other substrates (regular and labelled FB_1, OTA and ZEN) was analysed to determine the spiked mycotoxin concentration in these samples.

Mycotoxin exposure

Fifty individual seven-day-old BSFL (originating from the BSF colony of Bestico B.V.) were placed on 25 g of the wet spiked substrate (moisture content 65%) in a rearing cup (SPL Life Sciences Co., Ltd., Gyeonggi-do, South Korea) and placed in a climate chamber. Climate chamber conditions were set at 28°C, 60% RH and a day/night rhythm of 12/12 h was provided (Meijer *et al.* 2021a). On the sixth and seventh day of exposure, respectively, 3.47 ± 0.02 g and 3.00 ± 0.04 g of water was added to all containers to compensate for water loss. Exposure ended on day seven, when the BSFL were separated from the residual material (*i.e.* left over substrate and frass), weighed, counted, washed, dried, weighed again and transferred to clean (non-spiked) substrate to allow gut cleaning. On day eight, the larvae were again washed and dried, weighed, placed in plastic bags, and frozen at -18°C.

The HF eggs used in this study were taken from the HF colony maintained at the Laboratory of Entomology (Wageningen University). The rearing process was identical to that described in *Chapter 3* (Niermans *et al.* 2023a). An egg clutch of 100 HF eggs was placed on top of 30 g of the wet spiked substrate (moisture content 60%) in each of the 100 mL rearing cups (Greiner Bio-

One GmbH, Kremsmünster, Austria) and placed in a climate cell at 25 ± 1°C, 65% RH and a day/night rhythm of 12/12 h. On the fifth day of exposure, larvae were separated from the residual material, and the same protocol as described above for the BSFL (weighing, washing etc.) was applied. The only deviation to the original HF protocol (Niermans *et al.* 2023a) was that the HFL could ingest clean (non-spiked) substrate for *ca.* five hours. The residual material of the spiked substrates and the clean substrate (BSF and HF) was weighed, collected and stored at -18°C until further analyses.

Extraction procedure

Before extraction, the frozen BSFL and HFL were ground under liquid nitrogen to obtain a fine powder. The samples were then stored at -80°C. Sample extraction of the residual material was performed in accordance with and in-house validated method (according to SANTE/12682/2019; (EC 2019b)) based on the QuEChERS method and a slightly adjusted protocol was used for the substrate and larval samples. The extraction protocol was the same as reported in Camenzuli *et al.* (2018) and similar for the substrate and larvae, with adapted amounts of 1 g of HF substrate or BSFL and 500 mg of the HF larval sample. Amounts of solvents and other materials used in the extraction procedure were adjusted accordingly. Finally, 200 µL of sample extract was added together with 200 µL water in a syringeless PTFE filter vial (Mini-UniPrep, Whatman, Marlborough, MA), capped, vortexed and stored at 4°C until analyses. The same larval- and residual extracts as prepared for LC-MS/MS analyses were used for analyses on the LC-Q-Orbitrap^{TM}-MS. In order to calculate the mycotoxin (metabolite) concentrations in the samples, a matrix matched calibration was prepared in extract of each of the control matrices (substrate, larvae and residual material). The ZEN metabolite α-ZEL was not present in the standard reference mixture used for this experiment, therefore, quantification was done based on an in-house calibration line (in solvent).

LC-MS/MS analyses

All samples (substrate, larvae and residual material) were analysed for concentrations of either AFB_1, FB_1, OTA, ZEN and related metabolites, depending on the treatment. The LC-MS/MS system consisted of a Waters Acquity injection and pump system (Waters, Milford, MA) and an AB Sciex QTRAP 6500 triple quad system equipped with an electrospray ionization (ESI) source which was operated in positive and negative mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Tables S1 and S2). LC separation was performed by an Acquity UPLC HSS T3 1.8 µm 100x2.1 mm column (Waters, Milford, MA).

The composition of the eluents used for the positive and negative ionization mode and the LC eluent gradient for both ionisation modes was the same as reported in Camenzuli *et al*. (2018), however, in the current study the initial conditions were restored at 10.5 min and the run ended at 12.5 min (Camenzuli *et al.* 2018). The flow rate was 0.4 mL/min, the column temperature was

40°C, and the injection volume 5 µL. The conditions set for ESI were as follows: spray voltage 4.0 kV/-4 kV, temperature, 400°C, Ion Source Gas 1 and 2 were both set at 50 arbitrary units.

HRMS analyses

The same larval- and residual extracts as prepared for LC-MS/MS analyses were used for analyses on the LC-Q-Orbitrap™-MS. Chromatography and the variable data-independent acquisition (vDIA) in positive and negative mode were performed as described by Zomer and Mol (2015). In addition to this method, measurements were performed with a full scan top 5 DDMS, a full scan (mass range 135 - 1000 m/z, resolution 70.000) combined with a top5 DDMS2 (resolution 17.500) with a dynamic exclusion time of 10 s, in positive and negative mode. The DDMS method was performed for one sample of each triplicate sample set (per treatment).

Data collection and analysis

Data on survival and larval biomass on control and mycotoxin-spiked substrates were collected by counting and weighing larvae at harvest, after seven (BSF) and five (HF) days of exposure to the substrates, and after washing and drying. The weight of the residual material was calculated as the total weight of the container after exposure, minus the weight of the empty container and the weight of the larvae at harvest. Survival and growth data of both experiments were statistically analysed by a Kruskal-Wallis test followed by a Dunn's Multiple Comparison test in which the data were compared with the solvent control substrates (significance level (α) of 0.05; GraphPad Prism v4). In one of the labelled OTA replicates, some BSFL had escaped during the trial and, therefore, survival data of this replicate were excluded from further data analyses. Figures were made in GraphPad Prism v4.

SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA) was used to analyse the concentration data of the mycotoxin (metabolites) obtained via the analyses with LC-MS/MS. Identification of peaks was performed according to criteria for identification and recovery set in SANTE/12682/2019 (EC 2019b). The recovery percentages of analysed compounds for each of the matrices (substrate, larvae and residual material) are shown in Table S3. Analysed concentrations were corrected for these recovery percentages, to include for possible uncertainties (extraction efficiency, matrix effect and LC-MS/MS analyses) and was done according to Regulation (EC) 401/2006 (EC 2006a). The samples taken of the BSFL substrate were dry, however, the insects were provided wet substrate (35:65 feed:water). Therefore, the analysed concentrations were corrected to reflect the actual weight of the substrate as provided to the insects. As the ZEN metabolite α-ZEL was not present in the standard reference mixture, no recovery could be calculated and, therefore, the analysed concentrations for this compound were not corrected for recovery. Sample homogeneity was determined by calculating the deviation between the aflatoxin concentrations in the aliquots collected from the regular AFB_1 substrate. Replicate five (HF substrate) was excluded from this calculation since the results

indicated a technical issues with this sample. LOD and LOQ values were determined for the used method and were based on a calibration in solvent and adjusted for the recovery (%) of the respective matrices (substrate, larvae and residual material; Table S4). LOD and LOQ were determined as the lowest concentrations that complied to the aforementioned criteria for identification, with as additional criteria that the signal to noise ratio (S/N) had to be ≥ 3 for the LOD, and ≥ 10 for the LOQ. Mycotoxin (metabolite) concentrations < LOQ were set at 0 for the mass-balance calculations.

A bio-accumulation factor (BAF), representing the ratio of the concentration in the larval body (wet weight) as compared to the concentration present in the initial substrate (wet weight), was calculated in order to determine whether bio-accumulation occurred in the insects. The molar mass balance was calculated by expressing the total molar mass of post-experiment compounds (larvae + residual material) as a percentage of the mass of pre-experiment compounds (substrate). The calculation was performed for the regular substrates only, because reference standards were only available for metabolites of the regular mycotoxins. Molar mass balance calculations were performed for all substrates other than the control substrates. Detected concentrations from the LC-MS/MS analyses were adjusted for the wet mass measured for each of the treatments. The molecular weight of the parent compounds and metabolites used for the molar mass balance calculations are shown in Table S5.

In addition to the LC-MS/MS analyses, HRMS analyses was performed. Raw data files of the HRMS analyses were analysed by Compound Discoverer™ 3.2 (ThermoFisher Scientific, Waltham, MA, USA) to investigate the presence of modified mycotoxins or unknown metabolites. For each of the four spiked mycotoxins, the isotope labelled and regular samples were analysed together with controls. The Compound Discoverer™ workflow includes the selection of spectra, aligning of retention times, detection of compounds, grouping of compounds (labelled and regular (native) spiked samples were processed together with their respective controls), filtering of background noise (signal should be five times higher than the control samples), performance of a ChemSpider and mzCloud library search, and the prediction of elemental compositions (Figure S1). The workflow resulted in a list of potential compounds for which the software automatically generated molecular formulas, which were used for further analyses.

The isotopic label status (label only present in the labelled samples and absent in the regular ones) and a similar peak intensity of both the labelled and regular spiked samples were compared to the control samples, and used as additional criteria for identification. Only signals for which the isotope label was present in the labelled samples, as opposed to their regular counterparts, were manually selected. Subsequently, the peak intensity of labelled and regular samples, should be maximally a factor 5 difference. However, for most metabolites the difference was below a factor of 2. MS/MS spectra of the signals which met all aforementioned

criteria were checked for indications for the identification which includes looking for fragments and neutral losses corresponding to the respective mycotoxin fragmentation patterns. The use of isotope-labelled mycotoxins in this experiment provides additional confidence that detected signals with correct labelling status are metabolites of the mycotoxins. Unfortunately, the use of isotope labels only slightly enhanced the possibilities for molecular structure elucidation. To accurately describe the identification confidence of the compounds the method developed by Schymanski was used (Schymanski *et al.* 2014).

Results

Control substrate and sample homogeneity

The control substrates were analysed to determine the presence of possible mycotoxin background levels. The mycotoxins included in this study and their metabolites were absent (< LOD) from the two control substrates. For both the AFB_1 spiked substrates, the analysed corrected concentrations were within an acceptable deviation range of ± 20% - confirming the homogeneity of the spiked substances in the substrate (Table 1, Table S6).

Larval survival and biomass

Average survival of BSFL varied from 94 to 100% between the treatments, whereas survival of HFL varied between 46 to 95% (Figure 1). The average individual larval biomass varied between the treatments from 57.6 to 84.2 mg for BSFL, and from 3.1 to 22.0 mg for HFL.

Larval biomass did not differ significantly between the different substrates for BSFL ($P = 0.994$) and HFL ($P = 0.106$). AFB_1, FB_1, OTA and ZEN concentrations - at around 0.1x (FB_1) and 1x the ML or GV for animal feed materials - did not significantly affect survival of BSFL ($P = 0.111$) or HFL ($P = 0.229$).

Mycotoxin (bio-)accumulation and metabolism

A low concentration of ZEN was found in the BSFL, whereas FB_1 was present in a much higher concentration in the larvae (217.7 ± 68.9 µg/kg). Additionally, no mycotoxin (metabolites) were detected in concentrations above the LOD in the HF larval body. BAFs were calculated in order to determine whether bio-accumulation occurred in the larval body. Since mycotoxin metabolites were absent from the initial substrates, only the concentration of the parent compound was included in the calculation of the BAF. For both BSFL and HFL, the BAF was (near) zero for almost all compounds (Table 2). Only for FB_1 in BSFL, the BAF was between 0.41 and 0.46, therefore bio-accumulation did not occur (BAF < 1). All BAF values were similar for larvae fed the regular and the labelled mycotoxin substrates (Table 2).

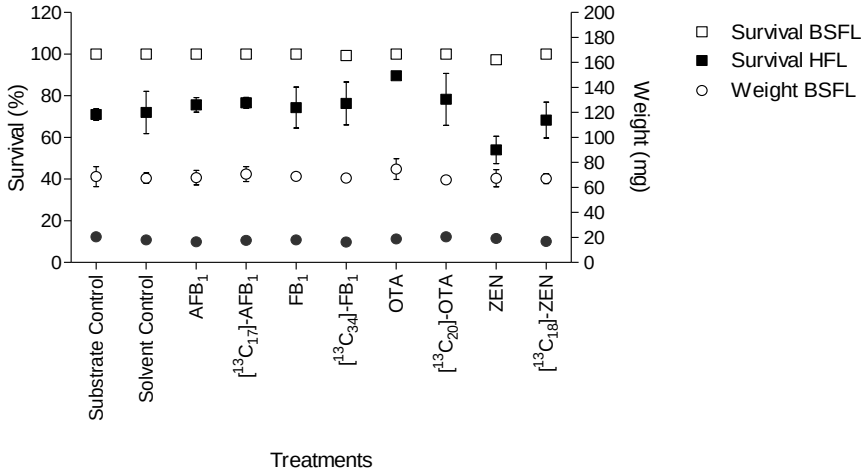

*Figure 1***.** Average larval survival and individual larval weight recorded after seven (BSFL) and five (HFL) days feeding on the included substrates. Error bars represent the SEM (n=3). Please note that deviations in some cases are zero/very small.

The average calculated molar mass balances for AFB_1 revealed 62% and 52% recovery for the BSFL and HFL treatments, respectively (Figure 2). In the residual material of the AFB_1-spiked substrates of BSFL, AFP_1 contributed for 13.5 ± 2.3% to the overall molar mass balance. None of the seven included aflatoxins were found in the substrate or residual material of the HFL experiment. Molar mass balances for FB_1 resulted in 72% recovery in the BSFL and 60% in the HFL experiment. Molar mass balances for the OTA treatments resulted in 109% (BSFL) and 105% (HFL) recovery. Recovery of OTA in BSFL and HFL is therefore considered complete. Parent compound and metabolites were present in the residual materials: especially OTα (89.2 ± 3.2%) contributed to the overall molar mass balance in the BSFL experiment.

In the HFL experiment, most OTA was found back in unchanged form; however, also OTα (13.2 ± 0.5%) and OTB (0.6 ± 0.02%) contributed to the overall recovery of OTA. Molar mass balances for ZEN showed 101% and 58% recovery for the BSFL and HFL treatments, respectively. In the substrates for the two insect species, a large proportion of ZEN was found back in unchanged form (BSFL 82.7 ± 2.9% and HFL 49.0 ± 1.2%), but also two metabolites of ZEN, *i.e.* α- and β-ZEL, contributed to the overall recovery. In the BSFL experiment, 15.2 ± 4.8% and 2.7 ± 0.9% of the overall molar mass balance was covered by α- and β-ZEL. In the HFL experiment, α- and β-ZEL contributed for 4.4 ± 1.3% and 2.2 ± 0.1%, respectively.

Table 2. Average concentrations (µg/kg) ± SD of parent compounds in the body of BSFL and HFL, and bio-accumulation factor (BAF) based on concentrations in substrate. Concentrations of all other mycotoxin (metabolites) in the larval body, were below their respective LOD.

	Spiked compound	Concentration parent compound	BAF
BSFL	AFB_1	< LOD	0.00
	$^{13}C_{17}$-AFB_1	< LOD	0.00
	FB_1	217 ± 68.8	0.41
	$^{13}C_{34}$-FB_1	193 ± 74.9	0.46
	OTA	< LOD	0.00
	$^{13}C_{20}$-OTA	< LOD	0.00
	ZEN	7.7 ± 3.3	0.02
	$^{13}C_{18}$-ZEN	7.7 ± 3.0	0.01
HFL	AFB_1	< LOD	0.00
	$^{13}C_{17}$-AFB_1	< LOD	0.00
	FB_1	< LOD	0.00
	$^{13}C_{34}$-FB_1	< LOD	0.00
	OTA	< LOD	0.00
	$^{13}C_{20}$-OTA	< LOD	0.00
	ZEN	< LOD	0.00
	$^{13}C_{18}$-ZEN	< LOD	0.00

Unknown metabolites

No new metabolites were found in the larval material of the AFB_1, FB_1 or OTA spiked substrates of the BSFL experiment, nor were new metabolites found in the larval material of the ZEN spiked substrate in the HFL experiment. Additional metabolites were found in BSFL reared on the ZEN spiked substrates. These metabolites are clearly ZEN metabolites as confirmed by their labelling status, and retention times were as expected considering the compositional change. Additionally, the fact that they fragmented to masses also present in the ZEN spectrum, indicated that they are ZEN metabolites. Table 3 mentions the details of the presumed new ZEN metabolites, hereafter named metabolite 1 and 2. The MS/MS spectra of metabolites 1 and 2 are shown in Figures S2a and S2b, respectively.

No new metabolites were found in the residual material of the AFB_1, FB_1, OTA and ZEN treatments in the BSFL experiment. However, in the residual material of the ZEN-spiked substrates in the HFL experiment, two additional metabolites were detected. Multiple molecular formulas seemed to fit the isotope patterns, and the MS/MS spectra provided limited information on the elemental composition and structure of the metabolites. Therefore, it is not possible to report an unambiguous molecular formula. Table 3 mentions the details of the presumed new ZEN metabolites, hereafter named metabolites 3 and 4.

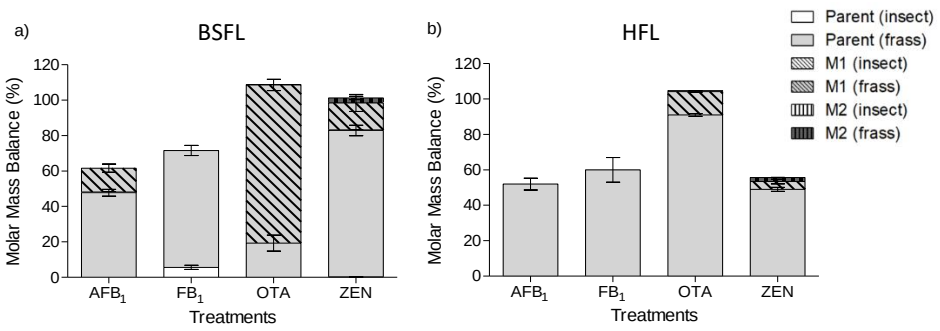

*Figure 2***.** Molar mass balance of AFB_1, FB_1, OTA and ZEN for a) BSFL and b) HFL fed on a mycotoxin spiked substrate. Error bars represent the SEM (n=3). Metabolite(s) (M) contributing to the mass-balance for AFB_1 was M1: AFP_1 ; for OTA were: M1: OTα, M2: OTB and for ZEN were M1: α-ZEL and M2: β-ZEL. An overview of the average contribution of the included mycotoxin (metabolites) can be found in Table S7.

The MS/MS spectrum of metabolite 3 is shown in Figure S3a. Due to the low intensity of the signal of metabolite 4 (mass 400.2437 Da), no high quality DDMS2 spectrum is included; instead a DIA (composite) spectrum of all ions between 295 and 405 m/z at the retention time of this metabolite is presented (Figure S3b). For both metabolites no fragments, except for 112.985 m/z, are visible which are also present in the spectrum of ZEN. Still, the presence of the isotope labelled-compounds in the labelled samples and the absence of labelled compound in the regular spiked samples, leads to the conclusion that these are ZEN metabolites.

*Table 3***.** Features detected in 'expected compound search' after filtering and evaluation, for the BSFL and HFL experiment.

Metabolite #	Matrix	Confidence level [a]	Predicted formula	Mol. mass (Da)	Composition change	Rt (min)
1	BSFL	4	C34H41NO6	559.2937	+ C16H19NO	10.896
2	BSFL	4	C44H64N2OP2	698.4519	+ C26H42N2P2 -O4	10.941
3	HFL residual material	5	-	282.02449	-	8.613
4	HFL residual material	5	-	400.2437	-	10.825

[a] Confidence level of identification is based on the Schymanski method (Schymanski *et al.* 2014).

Discussion

The current study showed that substrates contaminated with AFB_1, FB_1, OTA or ZEN fed to BSFL and HFL did not significantly affect larval survival or larval biomass. This is in line with other studies that showed that substrates spiked with AFB_1 or ZEN, and OTA, in concentrations similar

to and higher than tested in the current study, did not significantly affect survival and larval biomass of BSFL (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Leni *et al.* 2019) and HFL (*Chapter 3*; Niermans *et al.* 2023a).

In the BSFL experiment, the BAF was (near) zero for all tested compounds, except for FB_1 (BAF 0.41). FB_1 accumulation was also absent in a study in which BSFL were fed naturally contaminated substrates containing FB_1 (in a similar concentration as in this study) and other mycotoxins (Leni *et al.* 2019). Camenzuli *et al*. (2018) detected α- and β-ZEL in BSFL, in concentrations either below or just above the LOQ (Camenzuli *et al.* 2018). While, in the current study, none of the included mycotoxin metabolites were found in BSFL in concentrations above the LOQ. Furthermore, no mycotoxin (metabolites) were found in HFL, which is in accordance with a previous study (*Chapter 3*; Niermans *et al.* 2023a). The concentration of the mycotoxins determined in the insect body of BSFL in all treatments were far below the lowest GV for complementary and complete feed stuffs as set in EC Recommendation 2006/576/EC (EC 2006b) or below the lowest ML listed for aflatoxins (sum of AFB_1, AFB_2, AFG_1 and AFG_2) in a variety of foodstuffs (EC 2006c) and below the lowest ML for AFB_1 in feedstuff (EC 2002a), indicating no safety concerns for using these insects as feed. Currently, AFP_1, OTα, α- and β-ZEL are not considered in any of the European legislations with MLs or GV for the presence of mycotoxins in animal feed. To our knowledge, this is the first study to investigate accumulation of OTA metabolites in reared insects and, as such, no comparisons can be made.

The recovered fraction of AFB_1 in the molar mass balance of this BSFL experiment was higher (62%) than reported by Bosch *et al*. (2017) (4 - 17%) and Camenzuli *et al*. (2018) (11 - 17%) (Bosch *et al.* 2017; Camenzuli *et al.* 2018). In the study of Bosch *et al*. (2017), only AFB_1 and AFM_1 were included in the mycotoxin analyses (Bosch *et al.* 2017), while in the current study AFP_1 contributed largely to the molar mass balance, which could in part account for the difference. Additionally, exposure time in the studies of Bosch *et al*. (2017) and Camenzuli *et al*. (2018) was ten days (Bosch *et al.* 2017; Camenzuli *et al.* 2018), whereas in the current study exposure lasted for seven days. The possible roles of the insect itself, substrate microorganisms and photochemical degradation in mycotoxin degradation/metabolism, and the effect of this on the molar mass balance, needs to be investigated further. Additional research also needs to be performed on the degradation products and breakdown pathway of AFB_1 in HFL. So far, only one other feeding study with mycotoxins has been performed for HFL (*Chapter 3*; Niermans *et al.* 2023a). The proportion of AFB_1 recovered in the molar mass balance of both HF experiments were comparable, and AFB_1 metabolites did not contribute to the overall recovery.

Both *in vivo* and *in vitro* studies showed that glutathione S-transferases that catalyse the formation of GSH conjugates play a crucial role in the modulation of AFB_1 DNA-adduct formation (Allameh *et al.* 2000). Also in insects, phase I enzymes *e.g.* cytochrome P450's (Berenbaum *et al.* 2021; Meijer *et al.* 2019) and phase II enzymes *e.g.* glucosyltransferases (De Zutter *et al.* 2016)

are known to play a role in mycotoxin metabolism. Additional common metabolic mechanisms in insects include conjugation with glutathione, sulphate or glucose (Wilkinson 1986). No such conjugated metabolites were detected in this study. AFB_1 has been documented to bind to the N_7-position of guanine, one of the four DNA bases (Coskun *et al.* 2019). Such guanine-conjugates have molecular masses of 480 – 503 Da and the current method should have detected these since the upper limit of the full scan was 1000 Da. None of the expected AFB_1 metabolites or conjugates had a mass exceeding this limit.

In this study, a missing fraction of 28% (BSFL) and 40% (HFL) was calculated for FB_1. The same missing fraction (28% FB_1) was also found when BSFL were reared on a substrate naturally contaminated with FB_1 (in a similar concentration as in this study) in a mixture with other mycotoxins, such as DON and fumonisin B_2 (Leni *et al.* 2019). For HFL, the molar mass balance for FB_1 has not previously been calculated in other experiments. Analytical standards of FB_1 metabolites were not included in the analyses, as they were not available, but HRMS analyses did not lead to the discovery of (known or novel) FB_1 metabolites. FB_1 is a highly polar compound, resulting in a low absorption in humans and animals and a half-life of only a few hours (EFSA 2018a). When absorbed, FB_1 is rapidly cleared from systemic circulation of ruminants, pigs and avian species (EFSA 2018b). In mammalian species, FB_1 is known to be biotransformed to only a limited extent and the main phase I modification described in the literature entails the hydrolysis of ester groups leading to the formation of hydroxy-FB_1 (HFB_1) or p-hydroxy-FB_1 (EFSA 2018b). In ruminants (Rice and Ross 1994) and pigs (Fodor *et al.* 2008) extensive biotransformation of FB_1 into its hydrolysed forms has been observed. Further metabolism of FB_1 and its hydrolysed forms has been described *in vitro* (Seiferlein *et al.* 2007) and in rodents (Harrer *et al.* 2015), and includes *N*-acylation with fatty acids of various chain lengths (EFSA 2018a). Extractions and analyses of FB_1, HFB_1 and other modified forms should be similar and are often detected in the same chromatographic run (EFSA 2018b). It is plausible that, when present, the currently used extraction and detection methods were capable of extracting and detecting hydrolysed FB_1 metabolites as well. *N*-acyl metabolites are, however, often less polar due to their conjugation with long-chain fatty acids (EFSA 2018a) which might make co-extraction less effective.

Recovery of OTA in a previously performed study (41 - 62%) (Camenzuli *et al.* 2018) was substantially lower than in this study (109%). In the current study, the metabolite OTα contributed for a large proportion to the recovery in the mass balance for the BSFL experiment, which may explain the differences between these two studies, as OTα was not analysed in the other study. This is the first time a molar mass balance was calculated for OTA in an HFL experiment, so no comparison with other studies can be made. OTα and OTB both contributed, of which OTα even more so than OTB, to the overall recovery in both the BSFL and the HFL experiment. Literature shows that *in vivo* biotransformation of OTA seems to be low and mainly restricted to hydrolysis of the amide bond leading to OTα (EFSA 2020b), which seems to

correspond with the results of the current study. OTB has been reported as a minor metabolite (1% of OTA) *in vitro* and *in vivo* (EFSA 2020b). Current knowledge on comparative toxicity between OTA and OTB needs to be strictly differentiated between *in vivo* and *in vitro* effects. *In vivo*, OTB is clearly less toxic than OTA as tested in a variety of animal models. This lower toxicity could be explained by its lower affinity for plasma proteins and the fact that OTB seems to be more readily eliminated. *In vitro*, both OTA and OTB showed a similar acute cytotoxicity, provided that similar amounts are taken up and are intracellularly bound (Heussner and Bingle 2015). OTα, on the other hand, is approximately 100-fold less toxic than OTA (Heussner and Bingle 2015). The predominant conversion of OTA into OTα in both the HFL and BSFL experiments seems therefore favourable because of its lower toxicity.

The results of the current study for ZEN in relation to BSFL are largely similar with those reported by Camenzuli *et al.* (2018). HRMS analyses resulted in the detection of two new ZEN metabolites in BSFL. These metabolites could be identified with a confidence level of 4 resulting in the assignment of a molecular formula, but current spectral data provided insufficient evidence to propose an actual structure (Schymanski *et al.* 2014). The proportions of ZEN recovered in the molar mass balance in the current and another HFL study (*Chapter 3*; Niermans *et al.* 2023a) were comparable. Also, the ZEN metabolites α- and β-ZEL contributed to the molar mass balance in both HFL studies. Two additional ZEN metabolites were detected in the residual material of HFL. The spectral data provided limited information on the elemental composition and structure of the metabolites, and it was therefore not possible to report an unambiguous molecular formula (confidence level 5) (Schymanski *et al.* 2014). Quantification of these four novel metabolites was not possible because standards are not available, but based on the small peak area it could be concluded that these metabolites were either only present in very low concentrations or that the small peak area was caused due to a low extraction efficiency or ionisation issues. It therefore seems unlikely that they can explain the missing fraction in the molar mass balance of the spiked ZEN treatments.

Data obtained in this study indicate that a certain proportion of the tested mycotoxins has not been recovered. It was hypothesized that the mycotoxins present at the start of the experiments would have metabolized to unknown metabolic products in either the insect or the residual material. However, the current study indicated that this was not the case. As LC-MS/MS analysis entails a targeted method for known compounds with optimized settings for the compounds of interest, it is much more sensitive than the HRMS method. The HRMS method is, on the other hand, a generic method which can be used to collect data on a wide range of compounds, which at the same time limits its sensitivity for specific compounds. Furthermore, it is possible that the QuEChERS-based extraction method was not optimal for some of the metabolites that may have been present, like the fumonisin *N*-acyl-metabolites or DNA adducts, which could consequently have not- or incompletely been extracted leading to a reduced recovery. Larger or more polar

molecules, as for example, metabolites conjugated with glucoside or sulphate might not be extracted as effectively in the currently used (extraction) method. Analyses of the water phase obtained from the QuEChERS partitioning step, the addition of a hydrolysis step or enzymatic treatment to free metabolites bound to proteins, peptides or lipids, might provide more information on this.

Overall, data obtained in this study indicate that the parent mycotoxins and related metabolites are (largely) absent from the insect samples of both species, and present to some extent in the frass. In certain cases, a proportion of the tested mycotoxins has not been recovered, as seen from the incomplete mass balances. Based on the provided data it seems possible that additional (unknown) metabolites are responsible for this missing fraction, as we might have not extracted them with the used methods. Furthermore, follow-up *in vitro* studies assessing the toxicity of insects fed a mycotoxin-contaminated substrates could be helpful. Testing of BSFL and HFL fed a mycotoxin-contaminated substrate for mutagenicity or genotoxicity with for example an Ames test or a micronucleus test (EFSA 2011b) might be a suitable next step. Furthermore, it would be valuable to assess whether the current findings can be extrapolated to a more realistic scenario (naturally contaminated substrates containing multiple mycotoxins). Additionally, studies assessing the role of the larval specific metabolic enzymes, their microbiota and of microorganisms in the substrate in mycotoxin metabolism, complete breakdown of AFB_1 into CO_2 and possibly other small organic molecules, and photochemical degradation of mycotoxins in the substrate are recommended. To conclude, based on the findings presented here, the absence of mycotoxin accumulation in BSFL and HFL in levels above the set EU guidelines provides a positive outlook for the future use of these insects reared on mycotoxin-contaminated substrates as feed or food. Furthermore, our results suggest that mycotoxin-contaminated substrates which are otherwise deemed unsuitable for livestock consumption and therefore do not compete with commercial livestock feed, can be used as insect substrate without affecting growth and biomass provide economically interesting opportunities.

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Supplementary information

Table S1
Instrumental MS/MS parameters of mycotoxins analysed in positive ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
$[^{13}C_{17}]$-AFB_1	4.70	330.1	301.1	40	10	33	16
AFB_1 (ql)	4.70	313.1	128.1	40	10	91	10
AFB_1 (qn)	4.70	313.1	285.2	40	10	33	16
AFB_1 (ql2)	4.70	313.1	241.0	40	10	54	15
AFB_2 (ql)	4.40	315.1	259.2	40	10	43	18
AFB_2 (qn)	4.40	315.1	287.2	40	10	37	18
AFG_1 (ql)	4.10	329.0	200.0	40	10	53	12
AFG_1 (qn)	4.10	329.0	243.2	40	10	39	14
AFG_2 (ql)	3.80	331.1	245.2	40	10	43	14
AFG_2 (qn)	3.80	331.1	313.2	40	10	35	18
AFP_1 (qn)	4.20	299.0	271.0	40	10	33	18
AFP_1 (ql1)	4.20	299.0	114.9	40	10	71	12
AFP_1 (ql2)	4.20	299.0	90.9	40	10	67	10
AFQ_1 (qn)	3.70	329.1	310.8	40	10	29	16
AFQ_1 (ql)	3.70	329.1	177.0	40	10	45	22
AFQ_1 (ql2)	3.70	329.1	128.0	40	10	67	14
AFL (qn)	5.40	297.1	269.0	40	10	29	16
AFL (ql)	5.40	297.1	114.9	40	10	81	12
AFL (ql2)	5.40	297.1	141.0	40	10	65	14
AFM_1 (qn)	3.84	328.9	272.9	40	10	33	18
AFM_1 (ql)	3.84	328.9	229.0	40	10	55	16
$[^{13}C_{34}]$-fB_1	5.80	756.5	356.4	40	10	57	4
FB_1 (ql)	5.80	722.5	352.3	40	10	55	12
FB_1 (qn)	5.80	722.5	334.4	40	10	57	4
IS Caffeine ^{13}C pos	2.90	198.0	140.0	40	10	25	11
$[^{13}C_{20}]$-OTA	6.70	424.2	250.0	40	10	33	16
OTA (ql)	6.70	404.0	102.0	40	10	91	14
OTA (qn)	6.70	404.0	239.0	40	10	33	16
OTB (ql)	6.00	370.1	186.9	40	10	47	12
OTB (qn)	6.00	370.1	204.9	40	10	29	14

qn: quantifier ions. ql: qualifier ions

Table S2
Instrumental MS/MS parameters of mycotoxins analysed in negative ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
OTα (ql)	4.67	254.9	210.0	-30	-10	-22	-17
OTα (ql2)	4.67	245.9	166.9	-30	-10	-34	-19
OTα (qn)	4.67	245.9	211.0	-30	-10	-22	-17
[$^{13}C_{18}$]-ZEN	6.70	335.0	185.1	-175	-10	-32	-15
α-ZEL (ql)	6.45	319.2	130.0	-110	-10	-47	-20
α-ZEL (qn)	6.45	319.2	160.0	-110	-10	-41	-13
β-ZEL (ql)	6.00	319.3	130.0	-110	-10	-47	-20
β-ZEL (qn)	6.00	319.3	160.0	-110	-10	-41	-13
ZEN (ql)	6.70	317.1	131.1	-175	-10	-36	-11
ZEN (qn)	6.70	317.1	175.0	-175	-10	-32	-15

qn: quantifier ions. ql: qualifier ions

Table S3
Recovery per matrix (n=1 for substrate, larvae and residue), average recovery and within-lab reproducibility of the mycotoxin analyses in the BSFL and HFL experiment

Compound	$[^{13}C_{17}]$-AFB_1	AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1
BSFL substrate	76%	79%	83%	69%	-	-	-	-	-
BSFL	80%	81%	82%	84%	85%	88%	84%	72%	86%
BSFL residue	82%	83%	84%	84%	88%	90%	91%	92%	86%
HFL substrate	71%	68%	69%	73%	84%	73%	80%	79%	86%
HFL	70%	99%	73%	75%	75%	84%	75%	28%	76%
HFL residue	87%	88%	88%	87%	86%	86%	88%	89%	87%
Average recovery *	78%	83%	80%	79%	84%	84%	84%	72%	84%
RSD_{wR} *	8%	12%	9%	9%	6%	8%	8%	36%	5%

Compound	$[^{13}C_{34}]$-FB_1	FB_1	$[^{13}C_{20}]$-OTA	OTA	OTB	OTα	$[^{13}C_{18}]$-ZEN	ZEN	α-ZEL	β-ZEL
BSFL substrate	93%	88%	83%	85%	-	-	84%	81%	-	-
BSFL	95%	88%	74%	74%	74%	75%	88%	91%	-	86%
BSFL residue	106%	51%	88%	88%	86%	89%	92%	93%	-	92%
HFL substrate	99%	100%	92%	96%	77%	115%	88%	91%	-	78%
HFL	77%	73%	71%	74%	73%	56%	75%	75%	-	66%
HFL residue	111%	110%	90%	89%	89%	87%	99%	102%	-	90%
Average recovery *	97%	85%	83%	84%	80%	84%	88%	89%	-	82%
RSD_{wR} *	12%	24%	11%	10%	9%	26%	9%	11%	-	13%

* these values are calculated as based on a variety of different matrices including two insect species.

Table S4
Method LOD and LOQ (µg/kg) per mycotoxin in the different matrices

BSFL

Compound		AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1	FB_1	OTA	OTα	OTB	ZEN	α-ZEL	β-ZEL
Substrate	LOD	0.16	0.16	0.11	-	-	-	-	-	14.20	0.29	-	-	7.72	-	-
	LOQ	0.32	0.16	0.18	-	-	-	-	-	14.20	0.59	-	-	7.72	-	-
Larvae	LOD	0.15	0.16	0.09	0.15	0.45	0.31	0.55	0.31	14.20	0.34	1.47	0.74	6.87	5.00	5.81
	LOQ	0.31	0.16	0.15	0.59	1.00	1.05	1.83	1.02	14.20	0.68	4.40	1.49	6.87	10.00	11.63
Residue	LOD	0.15	0.16	0.09	0.14	0.44	0.29	0.43	0.31	24.51	0.28	1.24	0.64	6.72	5.00	5.43
	LOQ	0.30	0.16	0.15	0.57	0.98	0.97	1.43	1.02	24.51	0.57	3.71	1.28	6.72	10.00	10.87

HFL

Compound		AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1	FB_1	OTA	OTα	OTB	ZEN	α-ZEL	β-ZEL
Substrate	LOD	0.18	0.19	0.11	0.15	0.54	0.33	0.50	0.31	12.50	0.26	0.96	0.71	6.87	5.00	6.41
	LOQ	0.37	0.19	0.17	0.60	1.21	1.10	1.67	1.02	12.50	0.52	2.87	1.43	6.87	10.00	12.82
Larvae	LOD	0.13	0.18	0.11	0.17	0.47	0.35	1.41	0.35	17.12	0.34	1.96	0.75	8.33	5.00	7.58
	LOQ	0.25	0.18	0.17	0.67	1.05	1.17	4.71	1.16	17.12	0.68	5.89	1.51	8.33	10.00	15.15
Residue	LOD	0.14	0.15	0.09	0.15	0.46	0.30	0.44	0.30	11.36	0.28	1.26	0.62	6.13	5.00	5.56
	LOQ	0.28	0.15	0.14	0.58	1.02	1.00	1.48	1.01	11.36	0.56	3.79	1.24	6.13	10.00	11.11

Table S5
Molecular weight of parent compounds and analysed metabolites for mass calculation purposes.

Compound	Molecular weight (g/mol)
AFB_1	312.27
AFB_2	314.29
AFL	314.29
AFM_1	328.27
AFP_1	298.25
AFQ_1	328.27
FB_1	721.83
OTA	403.81
ZEN	318.36
OTα	256.64
α-ZEL	320.38
OTB	369.37
β-ZEL	320.38

Table S6
Concentration (µg/kg) of the AFB_1 measured in the different replicates of the initial BSFL and HFL substrates for homogeneity testing

Treatments/ Replicates	BSFL - AFB_1 (µg/kg)	HFL - AFB_1 (µg/kg)
1	21.2	22.5
2	21.7	27.5
3	22.0	27.9
4	21.5	26.6
5	22.3	-
6	25.1	26.4
7		28.7
8		27.6
9		26.8
10		29.4
Average	22.3	27.0
SD	1.3	1.3
RSD	6%	7%

Table S7
Overview of the average contribution of AFB_1, FB_1, OTA and ZEN and their metabolites to the overall molar mass balance (%)

		Larvae						Residual material							
	substrates	Parent compound		M1		M2		Parent compound		M1		M2		Total	
		(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM	(%)	SEM
BSFL	AFB_1	0.1%	0.01%	0.4%	0.04%			47.7%	1.9%	13.5%	2.3%			61.6%	1.8%
	FB_1	5.7%	1.2%					65.9%	2.9%					71.6%	6.2%
	OTA	0.1%	0.03%	0.002%	0.0002%			19.3%	4.5%	89.2%	3.2%	0.2%	0.005%	108.7%	1.9%
	ZEN	0.2%	0.04%	0.3%	0.04%	0.1%	0.02%	82.7%	2.9%	15.2%	4.8%	2.7%	0.9%	101.2%	6.5%
HFL	AFB_1							52.4%	3.4%					52.4%	3.4%
	FB_1							60.3%	7.0%					60.3%	7.0%
	OTA	0.005%	0.00002%					91.0%	0.7%	13.2%	0.5%	0.6%	0.02%	104.8%	0.4%
	ZEN					0.0004%	0.000004%	48.7%	1.2%	4.4%	1.3%	2.2%	0.09%	55.3%	2.2%

The metabolite contributing to the mass-balance for AFB_1 was M1: AFP_1. For OTA these were M1: OTα and M2: OTB, and for ZEN were M1: α-ZEL and M2: β-ZEL. This table shows the average contribution of the three replicates.

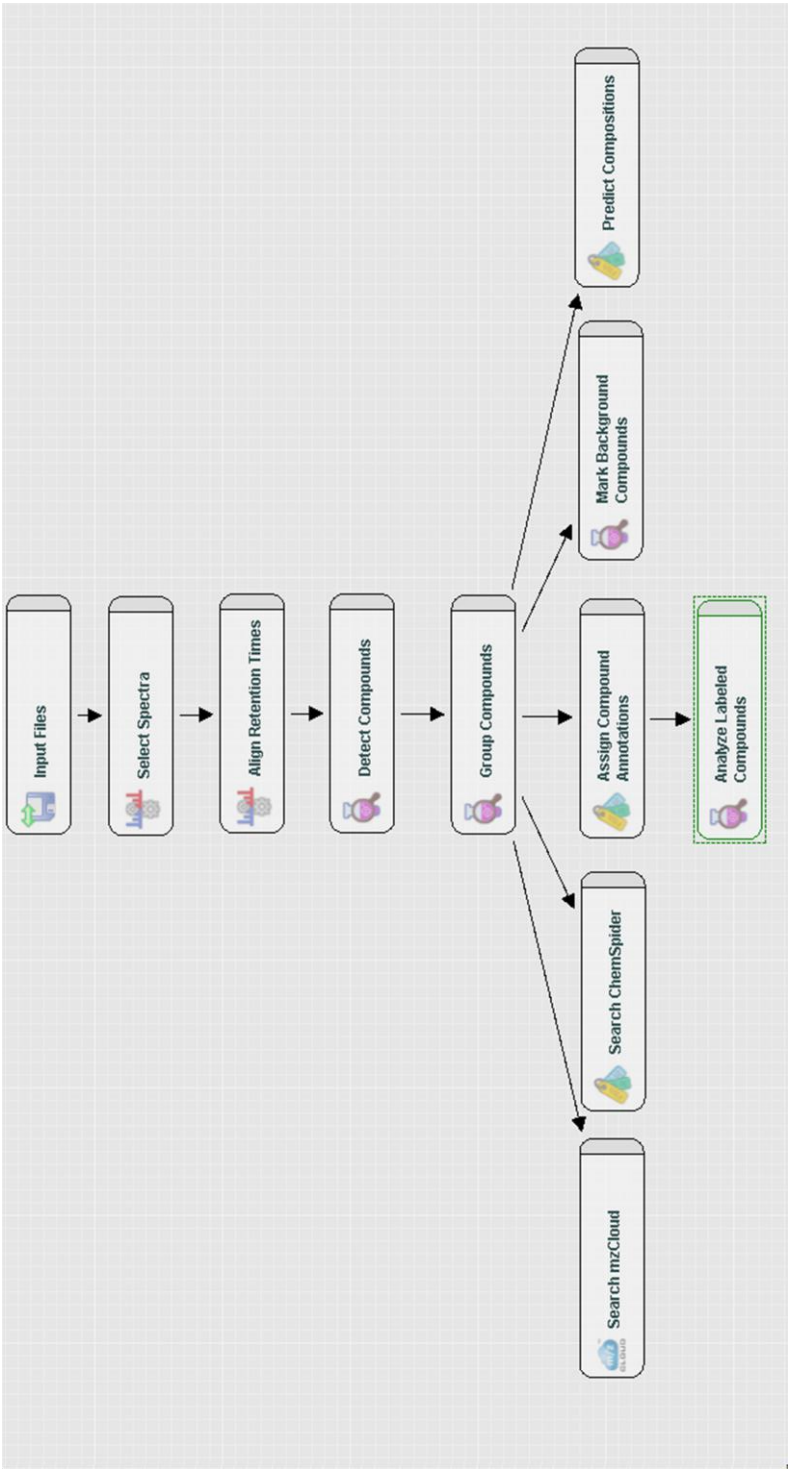

Figure S1
Compound Discoverer™ workflow

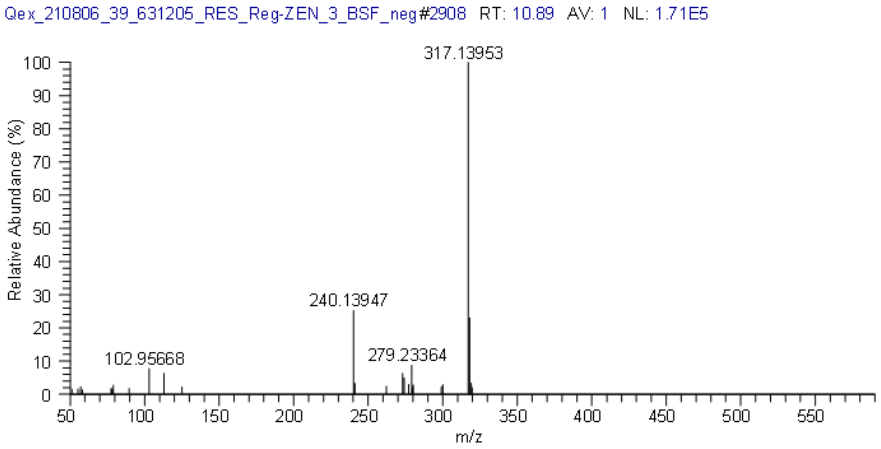

Figure S2a
MS/MS spectrum of metabolite 1, a presumed ZEN metabolite with a molecular mass of 559 Da in the BSFL experiment. The relative abundance on (Y-axis), quantifies the amount of an ion produced in relation to the amount of the base peak (most abundant ion which is set as 100%)

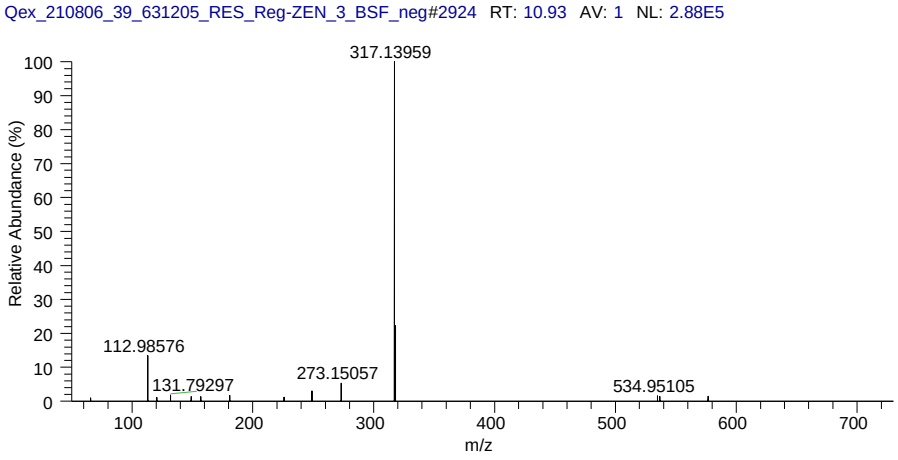

Figure S2b
MS/MS spectrum of metabolite 2, presumed ZEN metabolite with a molecular mass of 698 Da in the BSFL experiment. The relative abundance on (Y-axis), quantifies the amount of an ion produced in relation to the amount of the base peak (most abundant ion which is set as 100%)

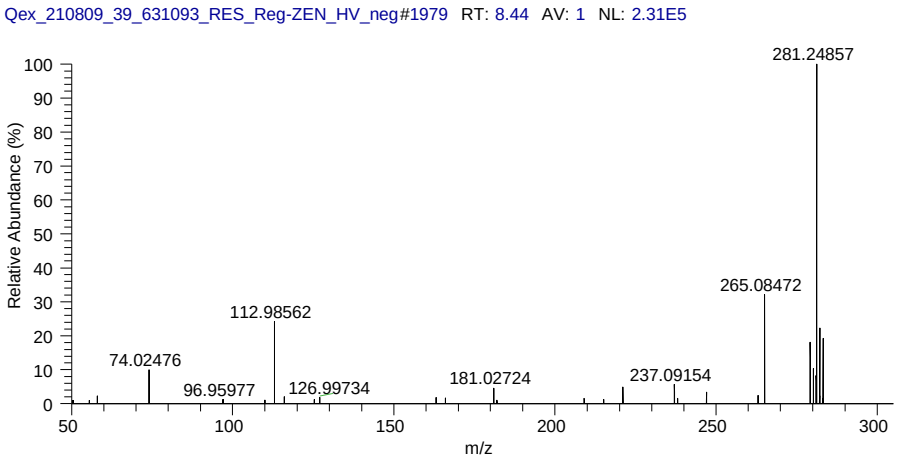

Figure S3a
MS/MS spectrum of metabolite 3, a presumed ZEN metabolite with a molecular mass of 282 Da in the HFL experiment. The relative abundance on (Y-axis), quantifies the amount of an ion produced in relation to the amount of the base peak (most abundant ion which is set as 100%)

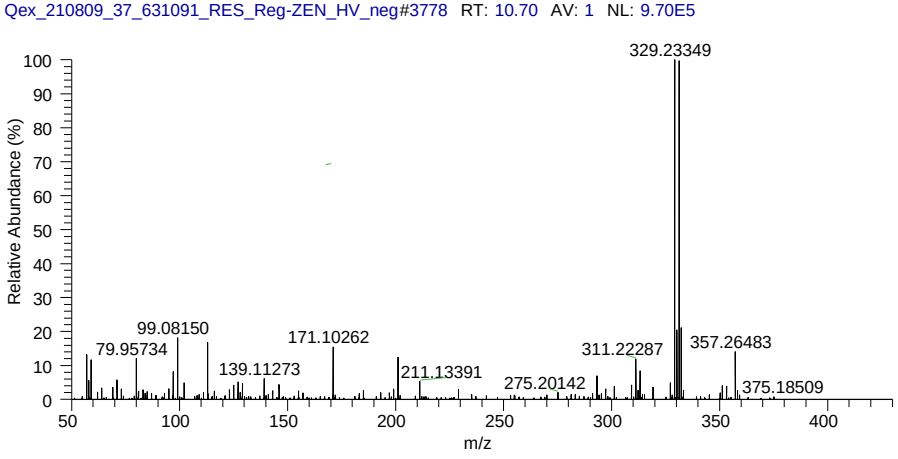

Figure S3b
DIA (composite) spectrum of metabolite 4, a presumed ZEN metabolite with a molecular mass of 400 Da in the HFL experiment. This DIA spectrum shows all ions between 295 and 405 m/z at retention time 10.70 min. The relative abundance on (Y-axis), quantifies the amount of an ion produced in relation to the amount of the base peak (most abundant ion which is set as 100%).

Chapter 5

The role of larvae of black soldier fly and house fly and of feed substrate microbes in biotransformation of aflatoxin B_1

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Abstract

Over the past few years, there has been growing interest in the ability of insect larvae to convert various organic side-streams containing mycotoxins into insect biomass that can be used as animal feed. Various studies have examined the effects of exposure to aflatoxin B_1 (AFB_1) on a variety of insect species, including the larvae of the black soldier fly (BSFL; *Hermetia illucens* L.; Diptera: Stratiomyidae) and the housefly (HFL; *Musca domestica* L.; Diptera: Muscidae). Most of these studies demonstrated that AFB_1 degradation takes place, either enzymatic and/or non-enzymatic, but the identification of a missing fraction in the molar mass balance indicated that part of the initially present AFB_1 could not be recovered. The possible role of feed substrate microorganisms (MOs) in this process has thus far not been investigated. This study aimed to investigate whether biotransformation of AFB_1 occurred and whether it is caused by insect-enzymes and/or by microbial enzymes of MOs in the feed substrate. Sterile- and non-sterile feed substrates were spiked with AFB_1 and incubated either with or without insect larvae (BSFL or HFL), and the AFB_1 concentration was recorded over time. We could not unravel whether non-enzymatic degradation of AFB_1 in the feed substrates occurred. The results showed that both BSFL and substrate-specific MOs play a role in the biotransformation of AFB_1 as well as in conversion of AFB_1 into AFP_1 and AFL, respectively. In contrast, HFL did not seem to contribute to AFB_1 degradation. The obtained results help in understanding the metabolism of aflatoxins by different insect species.

Introduction

Both the expected increase in the human population and the rise of the income levels will lead to a higher demand for food and nutrition, especially for animal proteins (UN 2015; Van Huis *et al.* 2013). Since the global area suitable for agricultural production is limited, the expected increase in animal protein production will likely be complemented with an increased environmental impact (Herrero *et al.* 2016). The increasing demand for alternative and sustainable proteins has led to the rise of insect farming. The emerging insect industry could contribute to food and feed security globally (Van Huis 2013).

A variety of insect larvae can be used to convert different organic side-streams into insect biomass for feed (Smetana *et al.* 2016). Larvae of the black soldier fly (BSFL; *Hermetia illucens* L.; Diptera: Stratiomyidae) and the housefly (HFL; *Musca domestica* L.; Diptera: Muscidae) are able to recycle these organic streams efficiently (Gold *et al.* 2018). However, organic side streams that could be of interest as a feed substrate for insect rearing could contain a variety of contaminants, among which mycotoxins. Mycotoxins are considered as an agriculturally relevant contaminant group affecting 60-80% of the food crops globally (Eskola *et al.* 2020). Mycotoxin contamination is not only associated with large economic losses and negative impacts on domestic and global trade, it also has a severe impact on the health of both animals and humans

(Eskola *et al.* 2020). Many different mycotoxins are known today (Berthiller *et al.* 2007), and from these aflatoxins have received most attention due to their presence and toxicity. Aflatoxins are secondary metabolites produced by fungal strains of the genus *Aspergillus* (Streit *et al.* 2012). Amongst the aflatoxins, aflatoxin B_1 (AFB_1) is most abundant in agricultural food crops (EFSA 2004). Due to its carcinogenic, teratogenic, mutagenic effects and immunosuppressive nature, maximum levels (ML) have been set for its presence in food (EC 2006c) and feed (EC 2002a) in Europe.

Multiple studies have examined the effects of AFB_1 exposure on a variety of insect species, among which BSFL (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; *Chapter 7*; Meijer *et al.* 2019; Niermans *et al.* 2023b; Niermans *et al.* 2023c) and HFL (*Chapter 3*; *Chapter 4*; Niermans *et al.* 2023a; Niermans *et al.* 2023b). Most of these studies calculated a molar mass balance, and used this as a measurement for the fraction of the initially present parent toxic compound (or metabolites) in the substrate that had been recovered in the larvae and the residual material. In the studies using AFB_1-spiked substrates, the molar mass balance was incomplete, indicating a missing fraction that was not recovered. Interestingly, when performing a feeding study with BSFL and peanut press cake naturally contaminated with a mix of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), the molar mass balance was close to being complete (*Chapter 7*; Niermans *et al.* 2023c). Furthermore, it is known that insects themselves possess the genetic machinery capable of metabolising mycotoxins (Berenbaum *et al.* 2021; *Chapter 2*; Niermans *et al.* 2021). However, thus far no study investigated the role of the substrate MOs versus the insect larvae in aflatoxin breakdown and metabolism.

In this study, we distinguish between enzymatic and non-enzymatic degradation. Enzymatic degradation is caused by the action of enzymes produced by living cells which causes a chemical reaction to happen resulting in the formation of metabolites. In non-enzymatic degradation, chemical reactions occur but enzymes are not involved. Enzymatic and non-enzymatic degradation of AFB_1 in the feed substrates for BSFL and HFL rearing has not been examined yet. However, multiple studies showed that biotransformation of AFB_1 after incubation in soil occurs (Accinelli *et al.* 2008; Angle and Wagner 1980; Juraschek *et al.* 2022), and it has been shown that biotransformation of AFB_1 in soil is mainly driven by soil specific microorganisms (MOs; Accinelli *et al.* 2008). Other studies have also shown the potential of (soil) MOs, especially bacteria, to enzymatically metabolise aflatoxins (Ji *et al.* 2016; Liu *et al.* 2022; Vanhoutte *et al.* 2016; Verheecke *et al.* 2016). The above mentioned studies clearly show that biotransformation of AFB_1 could take place in the insect substrate and that – in general –MOs are expected to play a role in this.

The aims of the current study were to I) investigate whether enzymatic and non-enzymatic degradation of AFB_1 in spiked feed substrates takes place, and to determine the role of II) the insect species BSFL and HFL, and III) the substrate-specific MOs (bacteria, fungi) in this process.

Materials and methods

Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and from Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and from TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

Insects

BSFL used in this study originated from the BSF colony of the Laboratory of Entomology, Wageningen University. Eggs were collected from the cardboard strips used as egg laying substrate, and larvae were reared on chicken feed (Kuikenopfokmeel, Kasper Faunafood, Woerden, the Netherlands) until seven days old. The colony is maintained in a controlled climate chamber (27°C, a relative humidity of 65% and a day/night rhythm of 16/8 h).

The HF eggs used in this study were taken from the HF colony reared at the Laboratory of Entomology, Wageningen University. This colony originates from the Faculty of Science and Engineering, University of Groningen. The HF rearing process was identical to that described in *Chapter 3* (Niermans *et al.* 2023a).

Spiked feed preparation

BSFL and HFL were exposed to a control feed substrate or a feed spiked with 20 µg/kg AFB_1. This selected concentration was based on the ML of AFB_1 allowed in all feed materials which is set in Directive 2002/32/EC (EC 2002a). Both feed substrate treatments were performed in quadruplicate. An overview of the included feed substrates can be found in Table 1.

To prepare the spiked feed substrate for BSFL, 1.9 mL of the AFB_1 spiking solution (in MeOH) was mixed with 618 mL water and 332 g chickenfeed (moisture content 65%; final concentration of 0.2% MeOH) to obtain a total of 950 mL spiked wet feed. The HFL spiked feed substrate was prepared by mixing 1.2 mL of the same spiking solution with 144 mL water, 216 mL Nipagin solution in water (final concentration: 0.9 mg/L; Merck, Darmstadt, Germany) and 240 g dry food mix (37% wheat bran, 56% wheat flour, 4% full fat milk powder (28.2 g fat/100 g) and 2% dry instant baker's yeast) to obtain a total of 600 mL spiked wet feed (moisture content 60%; final concentration of 0.2% MeOH). The control feed substrates were prepared in the same way, but without AFB_1, with MeOH in the same final concentration as added in the spiked feed substrates. In order to prepare the substrate for the sterilized treatment, the dry substrates for both the BSFL and the HFL were sterilized for 20 min at 121°C in a Vapour Line Lite Autoclave (VWR International, Radnor, Pennsylvania, US).

Table 1. Overview of experimental feed substrates

Species	Treatment code	With or without larvae	Type of matrix	Feed substrate	Replicates	Intended mycotoxin conc. (µg/kg ww)	Samples taken on day
BSFL	BSFC	With	Substrate	Control	4	0	1, 7, 9, 11, 13
	BSF+	With	Substrate	Spiked (AFB_1)	4	20	1, 7, 9, 11, 13
	BSF-	Without	Substrate	Spiked (AFB_1)	4	20	1, 3, 5, 7, 9, 11, 13
	BSFS	Without	Sterile substrate	Spiked (AFB_1)	3	20	1, 5, 9, 11, 13
HFL	HFC	With	Substrate	Control	4	0	1, 5, 7, 9
	HF+	With	Substrate	Spiked (AFB_1)	4	20	1, 5, 7, 9
	HF-	Without	Substrate	Spiked (AFB_1)	4	20	1, 3, 5, 7, 9
	HFS	Without	Sterile substrate	Spiked (AFB_1)	3	20	1, 3, 5, 7, 9

The BSFL substrate underwent the sterilization procedure twice, as pilot experiments showed that autoclaving once did not result in a sterile substrate. The same spiking procedure applied for the sterilized and non-sterilized treatments, however, instead of tap water, autoclaved MilliQ water was used. The wet feed substrates were mixed manually for 15 min.

In order to determine homogeneity of the non-sterile spiked feed, eight samples were taken. Homogeneity was confirmed when the measured concentrations (relative standard deviation of the replicates) in the eight samples differed ≤ 20% from each other. Four samples taken from the control feed were analysed to confirm the absence of aflatoxins in these feed substrates.

Experimental set-up

The spiked feed substrates were either incubated with the presence of BSFL or HFL (Table 1; BSF+ or HF+) or without larvae (Table 1; BSF- or HF-). Spiked sterilised feed substrates were prepared in order to study the effect of non-enzymatic degradation of AFB_1. Spiked feed substrates with- and without insect larvae allow a distinction between the contribution of biotransformation caused by the insect larvae or substrate-specific MOs, respectively.

For the treatments in which larvae were feeding on the substrate, 150 seven-days-old BSFL, with an individual average weight of 22.41 (± 0.19) mg were placed on 100 g of either the wet control- or wet AFB_1-spiked feed in a 480 mL rearing cup (BugDorm insect pots purchased from MegaView Science Co., Ltd., Taichung, Taiwan). The rearing cups were placed in a climate room at 27°C, relative humidity of 65% and a day/night rhythm of 12/12 h for seven days. 0.012 g HF eggs (corresponding to 200 eggs) were placed on 60 g of either the wet control- or wet spiked feed in a 480 mL rearing cup (BugDorm insect pots purchased from MegaView Science Co., Ltd., Taichung, Taiwan). The rearing cups were placed in a climate cell at 25°C, relative humidity of 65% and a day/night rhythm of 12/12 h for five days. At day seven, all BSFL were separated from the residual material (a mixture of left-over substrate and frass), washed, dried, weighed and transferred to clean (non-spiked) feed on the seventh day of exposure. This procedure was repeated on day eight of exposure after which the larvae were stored at -20°C until further analyses. The same procedure applied for HFL, however, here exposure ended after five days and larvae were fed on clean feed for ± 5.5 hours. For the feed substrates without larvae, samples of the residual material were taken at multiple timepoints (at least five) during the experiment. The same was done for the treatments with larvae, however, during the time that the larvae were feeding on the substrate no samples were taken in order to not interfere with the experiment.

The experimental set-up of the sterile substrate was adjusted slightly. For both the BSFL and HFL, only one batch of sterile spiked feed was prepared after which 5.8 (± 1.8) g was divided over fifteen tubes (sterile 50 mL centrifuge tubes, Greiner Bio-One GmbH, Alphen aan den Rijn, Netherlands). The caps of the tubes were sealed with Parafilm® and incubated under the same

conditions as mentioned previously. At each of the five timepoints (see Table 1), three tubes were collected, tested for sterility (see next section) and stored at -20°C until LC-MS/MS analysis.

Confirmation of sterility

Sterility of the substrate and residual samples collected for LC-MS/MS analysis was confirmed for each replicate at all sample collection days (see Table 1 for the exact days). Sterility was confirmed by plating sample extract on Luria-Bertani (LB) agar plates (Naveed *et al.* 2014; Zhang *et al.* 2011) and Sabouraud 4% Dextrose Agar (SDA; Millipore; Merck KGaA, Darmstadt, Germany (Kusari *et al.* 2012). The LB agar consisted Bacto™ Tryptone (final concentration 10 g/L; ThermoFisher Scientific Inc., Waltham, USA), Bacto™ Yeast Extract (final concentration 5 g/L; ThermoFisher Scientific Inc., Waltham, USA), NaCl (final concentration 5 g/L; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), Difco™ Agar (final concentration 15 g/L; Becton, Dickinson and Company, Franklin Lakes, USA) and MilliQ water. The SDA agar was prepared by mixing SDA (final concentration 65 g/L) and MilliQ water. Both were sterilized for 20 min at 121°C in a Vapour Line Lite Autoclave (VWR International, Radnor, Pennsylvania, US). After the agar cooled down to 60°C, the plates were poured and stored at 4°C until further use. One g of each of the collected samples at all timepoints was dissolved in two mL sterile PBS, vortexed for 30 s and set aside for 10 min to let the sediment settle. One-hundred µL of supernatant was pipetted on an agar plate (in duplo), spread with L-shaped sterile spreaders (Heathrow Scientific, Vernon Hills, US) on the agar plates. LB plates were incubated aerobically for 24 h at 27°C (Feizi *et al.* 2023) while the SDA plates were incubated aerobically at 25°C and were checked after 72 h and after 120 h (Fasuan *et al.* 2022). When the LB agar plates and SDA were free of colonies after incubation, samples were considered sterile (photos can be found in Figures S1 and S2).

Aflatoxin analyses

The extraction procedure for the substrate and residual material was performed as described by (Camenzuli *et al.* 2018). Extraction of the larval samples was performed with 0.5 g sample material, thus the extraction procedure *e.g*. volume of water (1.5 mL) and extraction solvent (2 mL) and magnesium sulphate (0.8 g) were adjusted accordingly. Aflatoxin concentrations in the samples were quantified by means of standard addition, therefore, each extract was prepared with- and without the addition of a standard mix containing eight aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFL, AFP_1, AFM_1 and AFQ_1). Two-hundred µL sample extract was mixed with either 190 µL water and 10 µL of the standard mix or only 200 µL water in a syringeless PTFE filter file (Mini-UniPrep, Whatman, Marlborough, MA). The files were capped, vortexed and placed in the refrigerator for 30 min after which the vials were closed and stored at 4°C until LC-MS/MS analyses.

The LC-MS/MS system consisted of a Waters Acquity injection and pump system (Waters, Milford, MA) and an AB Sciex QTRAP 6500 triple quad system equipped with an electrospray

ionization (ESI) source. LC-MS/MS analyses were performed exactly as described in *Chapter 7* (Niermans *et al.* 2023c). Aflatoxin analyses were also solely performed in positive ESI mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Table S1). LC separation was performed with an Acquity UPLC HSS T3 1.8 µm 100x2.1 mm column (Waters, Milford, MA).

Fungal identification

Identification of the present fungal species was done via MALDI-ToF analysis (Bruker MALDI Biotyper microflex LT-SH) after which the obtained spectra were matched against the specific MBT Filamentous Fungi module (MBT Fil. Fungi Library V5.0) and the regular MBT Compass Library Revision K (2022) inc. SR library: BTyp2.0Sec.Library 1.0.

Data analysis

LC-MS/MS data were analysed with SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA). Analysed concentrations were corrected for recovery percentages of analysed aflatoxins per matrix (Table S2). Detected concentrations from the LC-MS/MS analyses were adjusted for the wet weight measured for each of the treatments. Concentrations were calculated by dividing the peak area of the sample by the (area of the sample with standard addition minus the area of the sample) and multiplied by the addition level of the respective mycotoxin. Kruskal-Wallis tests (significance level α: 0.05) were performed in SPSS (IBM® SPSS® Statistics 28, New York, USA) to determine whether the AFB_1 concentration in the residue samples per timepoint differed significantly between treatments. Significance was calculated for the two insect species separately. All figures were made in GraphPad Prism v5.02. In the current study, statistical analyses were performed using the non-parametric Kruskal-Wallis test. This means that significance of a possible interaction between the AFB_1 concentration over treatment and time was not tested.

Molar mass balance calculations were performed for all AFB_1-spiked feed substrates. Detected concentrations from the LC-MS/MS analyses were adjusted for the wet weight measured for each of the treatments. Mycotoxin (metabolite) concentrations below the LOQ were set to zero. The concentrations (µg/kg) of the mycotoxins determined in larval biomass and mass of residual substrate by LC-MS/MS analysis were multiplied by the total wet weight of larval biomass or residual substrate mass, respectively, to yield the total amount of mycotoxin recovered, including the amounts of metabolites analysed. The molar mass balance was calculated by dividing the sum of the mycotoxin (metabolite) mass detected in the larvae and residual material by the mycotoxin molar mass, including that of metabolites quantified, present in the initial substrate on a wet weight basis. The molar mass balance was calculated for the residual material on either day seven (BSFL) or day five (HFL).

Results

Control feed substrates and sample homogeneity

None of the mycotoxins included in the analyses were detected (< LOQ) in the control feed substrates. For all feed substrates, the analysed corrected concentrations were within an acceptable deviation range of ± 20% , confirming the homogeneity of the spiked substances in the substrate (Table 2, Appendix G).

Aflatoxin metabolism

At day seven, samples of the BSF experiment were collected and a mass balance was calculated. The total aflatoxin (including AFB_1, AFL, AFP_1, AFM_1, AFQ_1) concentration in the BSFL was < LOQ, and the molar mass balance was therefore completely based on the aflatoxin concentration in the residual material. The total calculated molar mass balance in the BSFL experiment, with larvae (BSF+) was 50 ± 2.6% of which the aflatoxin metabolite AFP_1 contributed 11 ± 1.8%. The calculated molar mass balance for the treatment without BSFL (BSF-) was comparable as for the treatment with BSFL (60 ± 8.4%), and here not AFP_1, but AFL contributed (5 ± 2.5%) only to the overall mass balance (Figure 1). The molar mass balance for the sterile feed substrate (BSFS) was 73 ± 11.9%, and no AFB_1 metabolites were detected. An overview of the average contribution of the aflatoxins to the overall molar mass balance can be found in Table S5. The molar mass balance was significantly different ($P < 0.05$) when BSFL were present in the substrate (BSF+) as compared to the sterile substrate (BSFS).

Table 2. Overview of the analysed AFB_1 concentration in the spiked feed substrates (mean ± standard deviation) at the start (day 1) of the experiment

Treatment code	BSFC	BSF+	BSF-	BSFS	HFC	HF+	HF-	HFS
AFB_1 concentration (µg/kg ww)	< LOQ	20.2 ± 1.1	19.2 ± 0.7	18.5 ± 0.9	< LOQ	22.8 ± 2.3	22.2 ± 1.2	24.6 ± 3.3

Individual measurements for the replicates (n=4 for BSFC, BSF+, BSF-, HFC, HF+, HF-; n=3 for BSFS and HFS) can be found in Table S3. ww: wet weight; < LOQ: below limit of quantification. Table with overview of LODs and LOQ per respective matrix can be found in Table S4.

Data from the HFL experiment were collected on day five. The total aflatoxin concentration in the HFL was < LOQ. Additionally, none of the included AFB_1 metabolites contributed to the molar mass balance in the HFL experiment. Therefore, the molar mass balance was completely based on the recovery of AFB_1 in the residual material as compared to the concentration spiked in the feed substrate. The average calculated molar mass balance in the HFL experiment was similar for the treatments with (64 ± 3.5%) and without (69 ± 6.2%) larvae (Figure 1). The molar mass

balance for the sterile feed substrate was 78 ± 8.4%, and again solely based on AFB_1 itself. Similar to what was observed in the BSFL experiment, the molar mass balance was significantly less complete ($P < 0.05$) when HFL (HF+) were present in the substrate as compared to the sterile feed substrate (HFS).

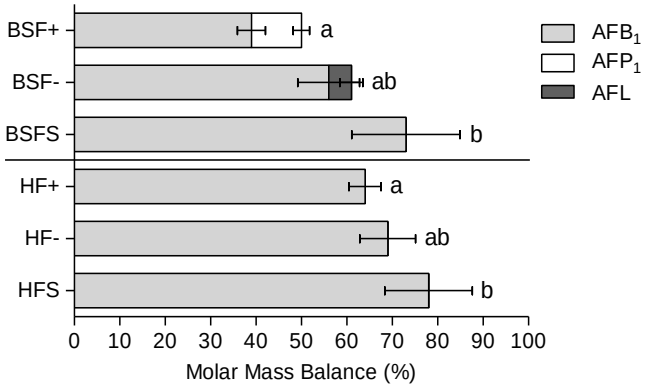
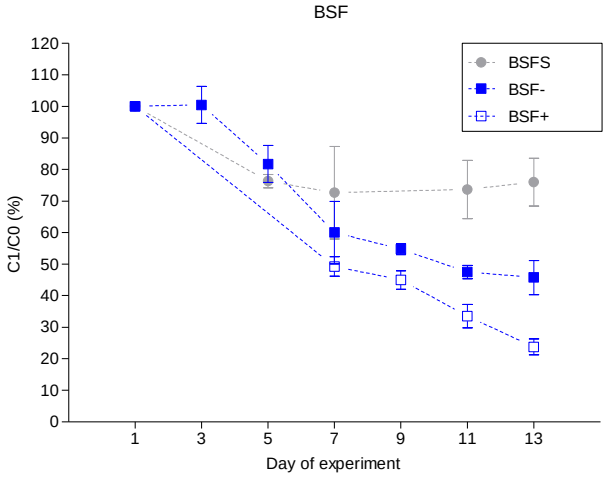
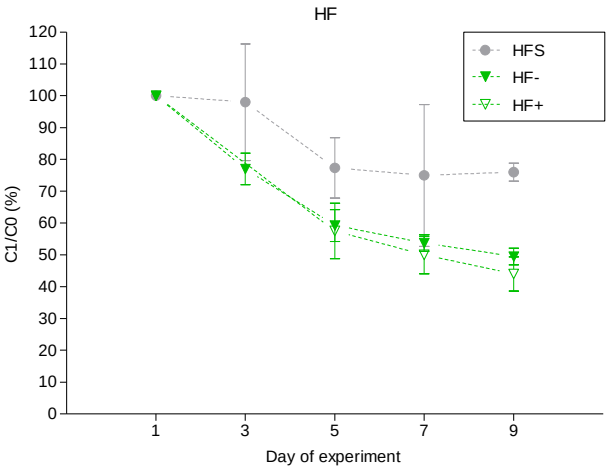


Figure 1. Molar mass balance of AFB_1 for BSFL and HFL fed on a AFB_1 spiked feed substrate with larvae (BSF+, HF+), for the spiked feed substrates on which no larvae had grown (BSF-, HF-) and for the sterile feed substrate (BSFS, HFS). Error bars represent the SD as a measure of variability between the replicates. Significance was tested separately for BSF or HF. Treatments with different letters differ significantly (Kruskal-Wallis, P = < 0.05).

Enzymatic *vs.* non- enzymatic degradation of AFB_1

The AFB_1 concentration in the BSFL feed substrates decreased over time finally resulting in a decrease of 76 ± 2% and 54 ± 5% for the non-sterile feed substrates with- and without BSFL, respectively, on the last day of the experiment (day 13). In the sterile feed substrate (BSFS), the AFB_1 concentration decreased with 24 ± 7% by the end of the experimental period. A significant decrease ($P < 0.05$) in AFB_1 concentration in the sterile feed substrate (BSFS) only occurred between the start of the experiment and day 5, afterwards the AFB_1 concentration remained unchanged until the end of the experiment ($P > 0.05$; Figure S3). The presence of BSFL in the feed substrate resulted in a lower AFB_1 concentration as compared to that in the sterile feed substrate (BSFS) from day seven, and in feed substrates without BSFL (BSF-) from day nine onwards ($P < 0.05$; Figure 2, Figure S4). The AFB_1 metabolite AFP_1 was formed in the BSF+ treatment and the absolute amount (µg) of AFP_1 – which was first found on day seven - decreased during the days that followed (Table 3).





	Day 5	Day 7	Day 9	Day 11	Day 13
BSF+	-	a	a	a	a
BSF-	a	ab	b	b	b
BSFS	a	b	-	b	b

	Day 3	Day 5	Day 7	Day 9
HF+	-	a	a	a
HF-	a	a	ab	ab
HFS	a	b	b	b

Figure 2. Percentage of total aflatoxin concentration (mean ± SD) over time as compared to the starting concentration (Day one; 100%) in the spiked feed substrate with larvae (BSF+ or HF+), the spiked feed substrates on which no larvae had grown (BSF-, HF-) and the sterilized feed substrate (BSFS, HFS). Larvae were removed from the feed substrate on either day seven (BSFL) or day five (HFL). A complete overview of the percentual decrease of AFB_1 for all feed substrates can be found in Table S6. Significance was tested separately for BSF and HF, and was done by comparing the treatments on each day. Treatments with different letters differ significantly (Kruskal-Wallis, P = < 0.05).

As the BSFL were removed from the residual material on day seven this could indicate that conversion of AFB_1 into AFP_1 stopped when the larvae were removed from the feed substrate. In the treatment without BSFL no AFP_1, but AFL was present from day seven onwards.

Table 3. Average and standard deviation of absolute amount (µg) of total aflatoxins, AFB_1, AFP_1 and AFL over time in the spiked feed substrate with BSFL and for the spiked feed substrates on which no larvae had grown

		Day 1	Day 7	Day 9	Day 11	Day 13
BSF+	AFB_1	2.03 ± 0.11	0.78 ± 0.06	0.72 ± 0.04	0.54 ± 0.05	0.38 ± 0.04
	AFP_1	< LOQ	0.21 ± 0.04	0.20 ± 0.05	0.13 ± 0.02	0.10 ± 0.01
BSF-	AFB_1	1.92 ± 0.07	1.07 ± 0.17	0.98 ± 0.09	0.84 ± 0.03	0.81 ± 0.12
	AFL	<L OQ	0.08 ± 0.06	0.07 ± 0.05	0.07 ± 0.05	0.07 ± 0.05

< LOQ: below limit of quantification. Table with overview of LODs and LOQ per respective matrix can be found in Table S4.

The AFB_1 concentration in the HFL feed substrates changed over time finally resulting in a decrease of 56 ± 5% and 51 ± 2% on day nine for the non-sterile feed substrates with- and without HFL, respectively. In the sterile feed substrate (HFS) the AFB_1 concentration decreased with 24 ± 3% by the end of the experimental period (day nine). The presence of HFL did not affect the time course of AFB_1 concentration ($P > 0.05$; all days) as compared to the non-sterile feed substrate without HF (HF-; Table 1, Figure S4).

Discussion

This study investigated whether enzymatic and/or non-enzymatic degradation of AFB_1 in spiked feed substrates takes place, and looked into the role of the larvae of two insect species, BSF and HF, and substrate-specific MOs (bacteria, fungi) in this process. In order to do so, we calculated a molar mass balance and tracked metabolism and degradation of AFB_1 over time in sterilised feed substrates and in non-sterilised feed substrates with or without larvae over time. In the current study no distinction can be made between the role of the insect metabolic systems and their gut MOs as only intact feeding insects were used. Therefore, the current study considers the insect and their gut MOs as inseparable entities, namely the insect system.

The calculated molar mass balance was not different for the treatments with BSFL (BSF+) and without (BSF). Around half of the initially present AFB_1, and a clear contribution of AFP_1 was found when calculating the molar mass balance in the BSF+ experiment. Total recovery of AFB_1 was slightly higher in *Chapter 4* (Niermans *et al.* 2023b), but the contribution of AFP_1 was similar. As AFP_1 was not formed in the treatment without BSFL (BSF-), we conclude that the BSFL caused its formation. Total recovery of aflatoxin was much lower in other studies (Bosch *et al.* 2017; Camenzuli *et al.* 2018) as compared to the current study. In the study of Camenzuli *et al*. (2018)

the concentration of AFP_1 was below the LOQ in the larvae and could not be determined in the residual material due to matrix interferences. In the study of Bosch *et al*. (2017) AFP_1 was not analysed, so no comparison between the AFP_1 contribution to the molar mass balance in the current study and those studies can be made. In the above two referred studies, BSFL were exposed to the AFB_1-spiked feed substrate for ten days, whereas in *Chapter 4* (Niermans *et al.* 2023b) and the current study, exposure lasted for seven days. Based on the collected data, it can be concluded that a longer exposure time to AFB_1 results in a higher fraction that is enzymatically metabolised by the larvae as well as the substrate MOs and, therefore, a more incomplete molar mass balance. The average calculated molar mass balance in the HFL experiment was similar for both treatments (with- and without larvae).

In the current study, the concentration of AFB_1 in both BSFL and HFL treatments (with- and without larvae) decreased over time. Metabolism of AFB_1 was more efficient in the presence of larval- and substrate MO-enzymes (BSF+) as compared to the substrate MOs alone (BSF-) from day nine onwards. AFB_1 was differently metabolised in treatments with- (AFP_1) and without BSFL (AFL), which indicated that both BSFL as well as substrate-inhabiting MOs seem to play a role in AFB_1 metabolism. However, for HFL, the absence of aflatoxin metabolites in the residual material, and the fact that the percentual decrease of AFB_1 over time was not affected by the presence of HFL in the feed substrates indicate that the HFL themselves do not have a role in AFB_1 metabolism.

In general, it can be stated that the substrate is the main determinant of the BSF larval gut bacterial community (Schreven *et al.* 2022). In addition, the larvae also alter the composition of the substrate bacterial community over time by inhibiting certain bacteria, changing the population sizes of resident bacteria, and by introducing gut bacteria into the substrate (Schreven *et al.* 2021). Schreven *et al*. (2022) showed that the presence of BSFL in chicken feed, used as a substrate for BSFL rearing, caused a change in the microbial composition of the substrate. While the chickenfeed was rich in *Curtobacterium* and *Pantoea* at the start of the study, *Pediococcus*, *Lactobacillus*, and *Weissella* were more dominant after five days of incubation with BSFL (Schreven *et al.* 2022). As chickenfeed was also used as a substrate in the current study, we assume that these shifts in MO community took place in the experiments reported here. We therefore assume that the treatment 'BSF-' did not trigger the same change in MO composition of the substrate as expected in the 'BSF+' treatment and might therefore have resulted in a lower degree of biotransformation by substrate MOs ($P < 0.05$; from day nine onwards) as compared to the treatments with BSFL. As the BSF larvae were removed from their feed substrate (BSF+) on day seven, this hypothesised shift in MO community in the feed substrate before day seven is likely responsible for the observed difference in the biotransformation of AFB_1 as compared to the BSF- and BSFS treatments from day nine onwards. The 'BSF-' treatment, however, clearly indicated the role of substrate-inhabiting MOs as AFL was

formed. Microorganisms, especially bacteria, have been studied for their potential to either metabolise mycotoxins or reduce their bioavailability (Abbès *et al.* 2013; Ben Taheur *et al.* 2019; Moretti *et al.* 2018; Peltonen *et al.* 2001; Topcu *et al.* 2010; Verheecke *et al.* 2016). For example, inoculation of 0.24 mg/kg AFB_1 with *L. plantarum PTCC* 1058 (37°C, 4-7 days, 9.10^9 CFU/mL) resulted in an AFB_1 reduction efficacy of 77% in corn (Verheecke *et al.* 2016). Additionally, Peltonen *et al*. (2001) examined the AFB_1 binding potential of twelve *Lactobacillus* strains and found that AFB_1 was rapidly bound (17.3 to 59.7%) by the respective bacteria (Peltonen *et al.* 2001). Authors hypothesised that AFB_1 can bind to the peptidoglycans and polysaccharides in the cell wall of lactic acid bacteria (Ben Taheur *et al.* 2019; Topcu *et al.* 2010). When AFB_1 is bound to the cell wall, the efficiency of its extraction can be influenced. It has, for example, been demonstrated that the extraction efficiency of AFB_1, when bound to *L. rhamnosus*, was dependent on the various conditions of the extraction method used, including the extraction solvent, pH, and incubation temperature, among others (Haskard *et al.* 2001).

Previous (vermicomposting) studies with HFL showed the potential of this insect species to alter the abundance and structure of the bacterial community in the feed substrate (Li *et al.* 2019; Zhang *et al.* 2012). However, in the current study no difference in AFB_1 reduction was observed for the HFL treatments. Schreven *et al*. (2022) showed that the impact of BSFL on microbial composition of the substrate is substrate-dependent and that bacterial communities in larvae and substrates can differ in composition depending on the feed substrate (Schreven *et al.* 2022). The use of the different feed substrates therefore result in different substrate-inhabiting and insect gut-associated MOs and possibly different AFB_1 metabolic pathways. Though studied for BSFL, this might also be applicable for HFL.

In the current study, AFB_1 enzymatic and non-enzymatic degradation was higher in the non-sterile substrate with insect larvae (BSF+, HF+) as compared to the non-sterile substrate without insect larvae (BSF-) and the sterile substrate (BSFS, HFS) on the final day of the experiment. The AFB_1 concentration in the sterile feed substrates (BSFS, HFS) was around 24% lower at the end of the experiment as compared to the starting concentration, which seems to indicate that non-enzymatic degradation took place in the sterile substrates. Aflatoxins are known to be photosensitive, however, their non-enzymatic degradation efficiency depends on UV intensity (Liu *et al.* 2010; Stanley *et al.* 2020). During the incubation step in the experiment, samples were not exposed to daylight and the UV-intensity of the fluorescent tubes present in the incubator was negligible. We therefore assume that, in the current experiment, UV exposure did not play a role in the non-enzymatic degradation of AFB_1. Furthermore, we cannot exclude that the AFB_1 which was spiked to the feed substrate bound to *e.g.* the matrix of plant DNA, proteins and other (macro)molecules present in the feeding substrates during incubation. Resulting from this, extraction of AFB_1 in the residual material may have been incomplete with the extraction method used. This unextracted AFB_1 could account for a part of the missing fraction in the molar

mass balance. Furthermore, we cannot exclude that conjugated metabolites were formed during the experiment. The used extraction method might not be optimal for extracting AFB_1 metabolites conjugated with *e.g.* glutathione. Addition of a hydrolysis step or enzymatic treatment to liberate bound AFB_1 and/or conjugated metabolites during extraction, and/or analyses of the water phase obtained from the QuEChERS partitioning step might be essential. Additionally, possible degradation of AFB_1 - an alteration of the molecular structure which includes metabolization and mineralization (complete decomposition into CO_2) - over time could explain this difference.

In the current study, sterile BSFL and HFL substrates were prepared. However, even though the BSFL substrate was autoclaved (20 min at 121°C) twice, growth of fungal colonies were still observed in the LB- and SDA agar plates. We attempted to identify the present fungal species via MALDI-ToF analysis, however, no match could be found using the available database. Fungal growth was not observed in all replicates collected on a day and neither on all days of the experiment. As the AFB_1 concentration did not differ much between replicates (with and without fungal growth), and because the remaining percentage of AFB_1 did not significantly differ between day seven, eleven and thirteen in the BSFL experiment we believe that this provides enough evidence that the presence of the fungal species did not have a role in AFB_1 breakdown in the current study.

Other studies have also shown the potential of MOs for biotransformation of other mycotoxins *e.g*. fumonisin B_1 (Liu *et al.* 2022), deoxynivalenol (Ji *et al.* 2016; Liu *et al.* 2022), and zearalenone (Ji *et al.* 2016; Król *et al.* 2018; Liu *et al.* 2022; Yi *et al.* 2011). As the current study only focused on AFB_1, we advise to perform a similar study in which the role of the insect larvae and the substrate-specific MOs and their potential mycotoxin degrading capacities are investigated for other mycotoxins.

Overall, the current study showed that both the BSFL system and substrate-specific MOs play a role in the biotransformation of AFB_1 as well as in conversion of AFB_1 into AFP_1 and AFL, respectively, whereas the HFL system did not seem to be involved. Different AFB_1 metabolism/degradation patterns can be expected for different feed substrates and/or insect species. To get a complete picture of AFB_1 enzymatic and non-enzymatic degradation in insect feed substrates, spiking with isotopically labelled standards and tracking the formation of labelled CO_2 is advised. Additionally, investigation of enzymatic and non-enzymatic degradation of AFB_1 and the role of both substrate specific MOs and insects in naturally contaminated materials might provide valuable insights. Furthermore, examination of the extraction efficiency of AFB_1 bound to the cell wall of substrate MOs, plant-derived matrix, and conjugated metabolites with an optimized extraction method might be essential. In conclusion, this study showed that substrate specific MOs as well as the BSFL play a role in enzymatic degradation of

AFB_1 while occurrence of non-enzymatic degradation of AFB_1 in the feed substrates cannot be confirmed. HFL themselves do not have a role in AFB_1 metabolism.

Acknowledgments

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Supplementary materials

Table S1

Instrumental MS/MS parameters of mycotoxins analysed in positive ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
AFB_1 (ql)	4.70	313.1	128.1	40	10	91	10
AFB_1 (qn)	4.70	313.1	285.2	40	10	33	16
AFB_1 (ql2)	4.70	313.1	241.0	40	10	54	15
AFB_2 (ql)	4.40	315.1	259.2	40	10	43	18
AFB_2 (qn)	4.40	315.1	287.2	40	10	37	18
AFG_1 (ql)	4.10	329.0	200.0	40	10	53	12
AFG_1 (qn)	4.10	329.0	243.2	40	10	39	14
AFG_2 (ql)	3.80	331.1	245.2	40	10	43	14
AFG_2 (qn)	3.80	331.1	313.2	40	10	35	18
AFL (qn)	5.40	297.1	269.0	40	10	29	16
AFL (ql)	5.40	297.1	114.9	40	10	81	12
AFL (ql2)	5.40	297.1	141.0	40	10	65	14
AFP_1 (qn)	4.20	299.0	271.0	40	10	33	18
AFP_1 (ql1)	4.20	299.0	114.9	40	10	71	12
AFP_1 (ql2)	4.20	299.0	90.9	40	10	67	10
AFM_1 (qn)	3.84	328.9	272.9	40	10	33	18
AFM_1 (ql)	3.84	328.9	229.0	40	10	55	16
AFQ_1 (qn)	3.70	329.1	310.8	40	10	29	16
AFQ_1 (ql)	3.70	329.1	177.0	40	10	45	22
AFQ_1 (ql2)	3.70	329.1	128.0	40	10	67	14

qn: quantifier ions. ql: qualifier ions

Table S2

Recovery of the mycotoxins in the different matrices (substrate, residue) analysed

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
BSFL substrate	94%	100%	99%	106%	104%	94%	103%	103%
BSFL residue	81%	81%	88%	86%	90%	89%	73%	76%
HFL substrate	81%	83%	81%	67%	87%	76%	90%	86%
HFL residue	86%	86%	92%	110%	97%	78%	94%	162%

Table S3
Overview of AFB_1 concentration (µg/kg) in the experimental feed substrates

Compound	BSFL feed substrate	HFL feed substrate
Control (1)	< LOQ	< LOQ
Control (2)	< LOQ	< LOQ
Control (3)	< LOQ	< LOQ
Control (4)	< LOQ	< LOQ
AFB_1 (1)	21.8	19.5
AFB_1 (2)	26.1	20.1
AFB_1 (3)	22.5	19.6
AFB_1 (4)	20.8	21.8
AFB_1 (5)	23.9	19.3
AFB_1 (6)	22.3	20.2
AFB_1 (7)	21.4	18.7
AFB_1 (8)	21.6	18.5
AFB_1 sterile (1)	19.5	24.8
AFB_1 sterile (2)	18.4	20.7
AFB_1 sterile (3)	17.6	26.9

The substrate was also analysed for the presence of the aflatoxins B_2, G_1, G_2, AFL, P_1, M_1 and Q_1 but none of them were detected. < LOQ: below limit of quantification

Table S4
Overview of the limit of quantification (LOQ) (ng/mL) per aflatoxin in the different matrices

Compound	AFB_1	AFL	AFP_1	AFM_1	AFQ_1
Substrate	< 0.2376	1.435	0.989	0.889	0.917
Residue	< 0.2376	1.082	0.688	0.822	0.946

Table S5
Overview of the mean contribution of the aflatoxins to the overall molar mass balance (%)

Treatments	AFB_1		AFL		AFP_1		AFM_1		AFQ_1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
BSF+	38.5%	1.5%	< LOQ		11.1%	1.8%	< LOQ		< LOQ	
BSF-	55.5%	3.4%	4.6%	1.3%	< LOQ		< LOQ		< LOQ	
BSFS	72.6%	6.9%	< LOQ		< LOQ		< LOQ		< LOQ	
HF+	64.5%	1.8%	< LOQ		< LOQ		< LOQ		< LOQ	
HF-	68.9%	3.1%	< LOQ		< LOQ		< LOQ		< LOQ	
HFS	78.1%	4.8%	< LOQ		< LOQ		< LOQ		< LOQ	

This table shows the average contribution of either three (BSFS, HFS) or four replicates (SF+, BSF-, HF+, HF-). < LOQ: below limit of quantification

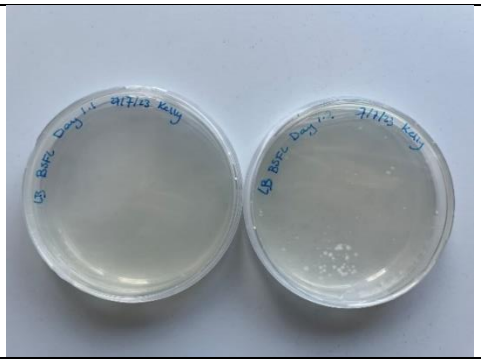
Table S6
Overview of the mean decrease (%) of the total aflatoxin concentration in the different treatments

Treatments	Day 3		Day 5		Day 7		Day 9		Day 11		Day 13	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BSF+					49%	3%	45%	3%	34%	4%	24%	2%
BSF-	101%	6%	82%	6%	60%	10%	55%	2%	47%	2%	46%	5%
BSFS			77%	2%	73%	15%			74%	9%	76%	7%
HF+			58%	9%	50%	6%	44%	5%				
HF-	77%	5%	59%	5%	54%	3%	49%	2%				
HFS	98%	18%	78%	9%	75%	22%	76%	3%				

This table shows the average contribution of either three (BSFS, HFS) or four replicates (BSF+, BSF-, HF+, HF-).

Figure S1

Pictures of Luria-Bertani (LB) agar plates and Sabouraud 4% dextrose agar (SDA) plates obtained during the BSFL experiment. Microbial growth in the substrate/residual material was investigated by plating sample extracts on LB and SDA plates. Samples were taken at day 1, 5, 7, 11, and 13. Two replicates per sample were incubated for 48 h (LB) or 120 h (SDA). The agar plates were photographed after incubation.



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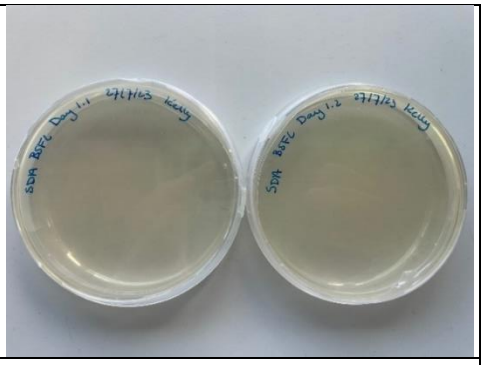

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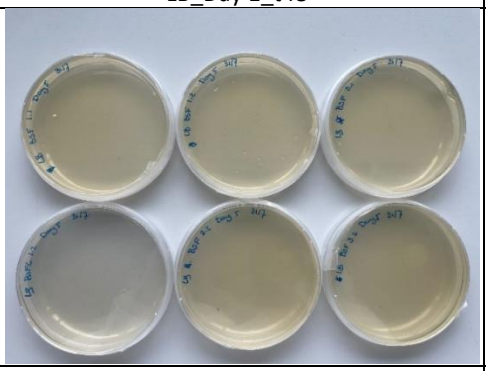

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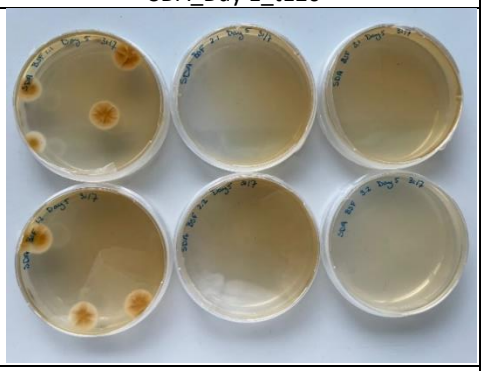

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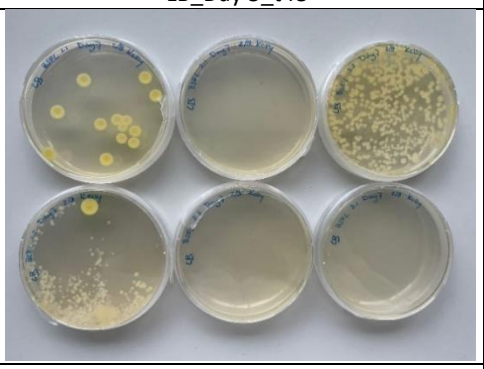

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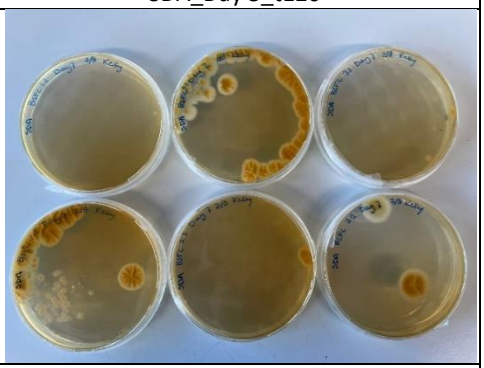


SDA_Day 7_t120

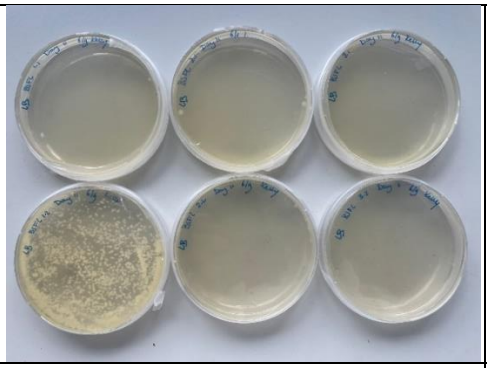
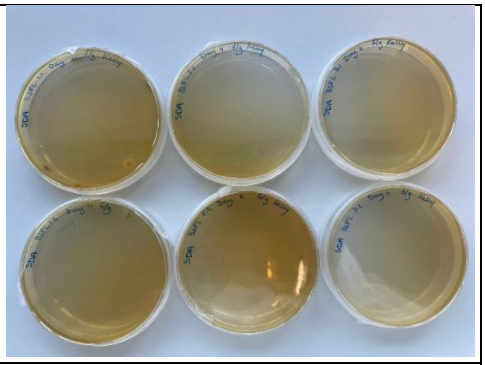
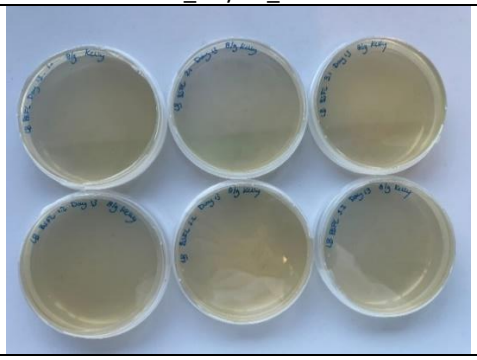
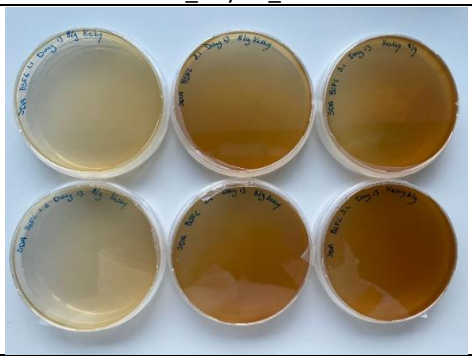
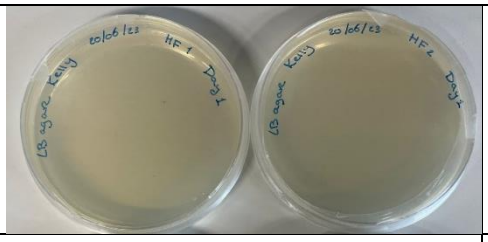
	
LB_Day 11_t48	SDA_Day 11_t120
	
LB_Day 13_t48	SDA_Day 13_t120

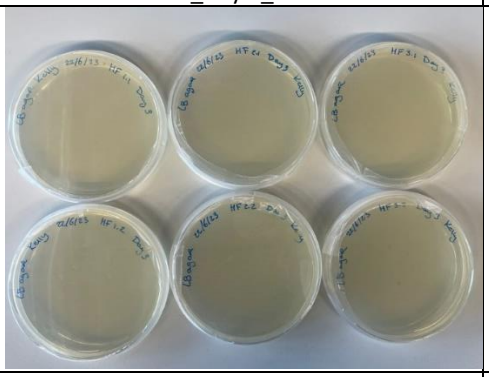
Figure S2

Pictures of Luria-Bertani (LB) agar plates and Sabouraud 4% dextrose agar (SDA) plates obtained during the HFL experiment. Microbial growth in the substrate/residual material was investigated by plating sample extracts on LB and SDA plates. Samples were taken at day 1, 3, 5, 7, 9. Two replicates per sample were incubated for 48 h (LB) or 120 h (SDA). The agar plates were photographed after incubation.



LB_Day 1_t48

SDA_Day 1_t120



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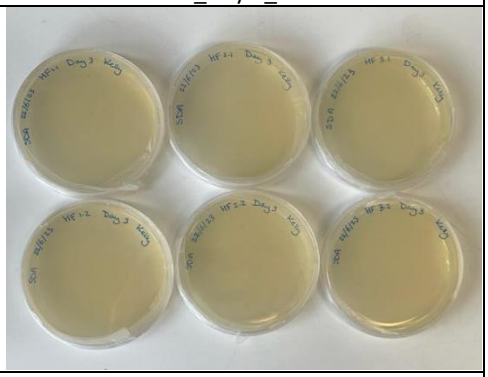

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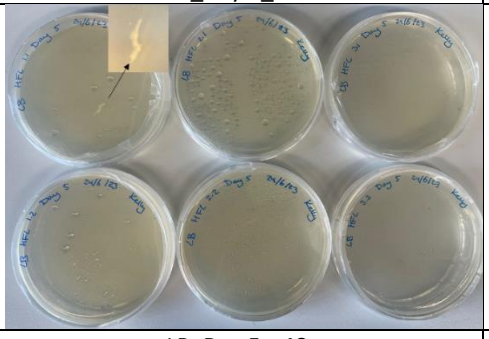

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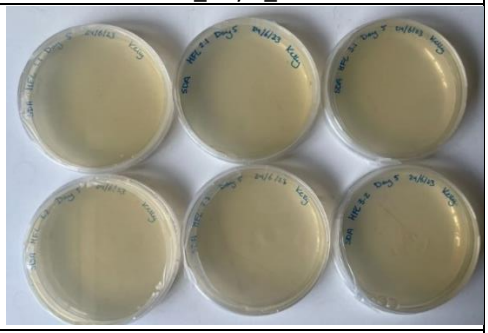

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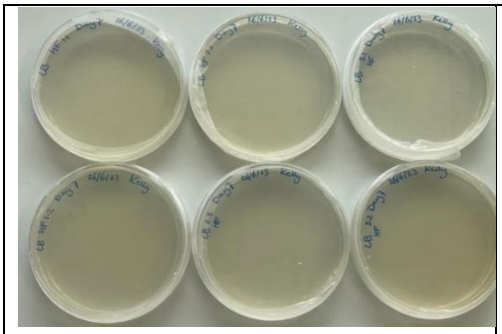	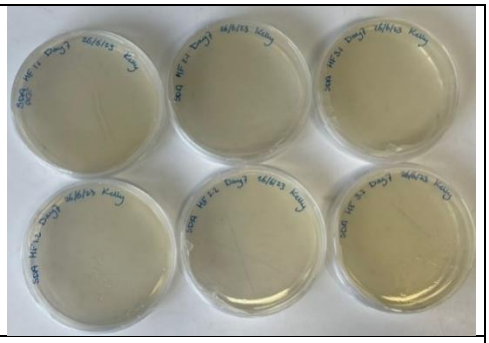
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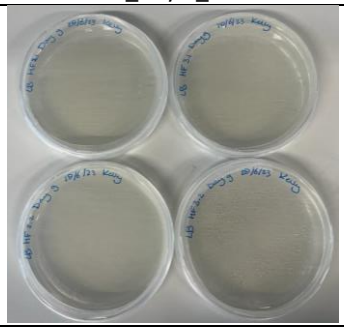	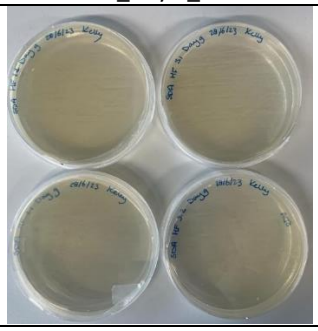
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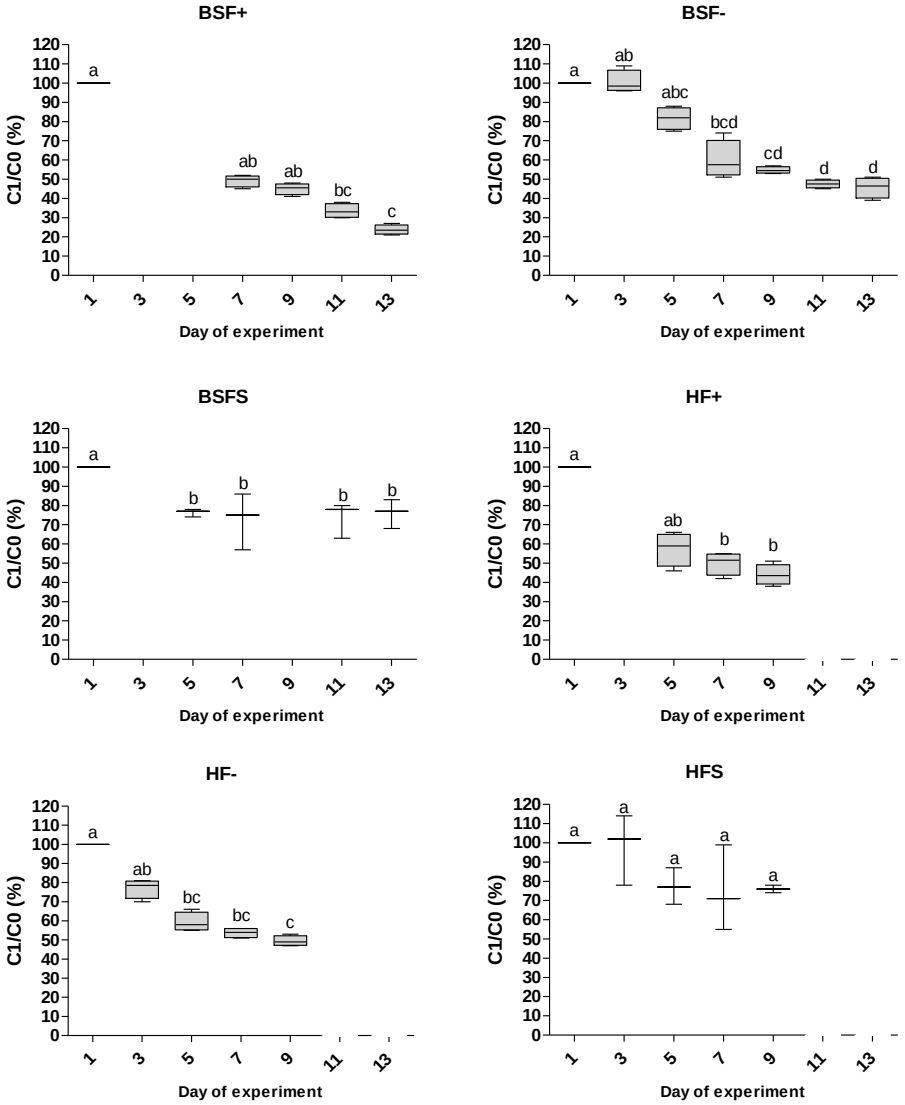

Figure S3

Whiskers plot: min to max. Effect of time within a treatment on enzymatic and non-enzymatic degradation of AFB_1 (Kruskal-Wallis, pairwise comparisons; P = < 0.05). Significance was calculated within experiments (BSF or HF) only, and was done by comparing the treatments on each day (n=4: BSFL+, BSFL-, HF+, HF-; n=3: BSFS, HFS). Treatments with different letters differ significantly.

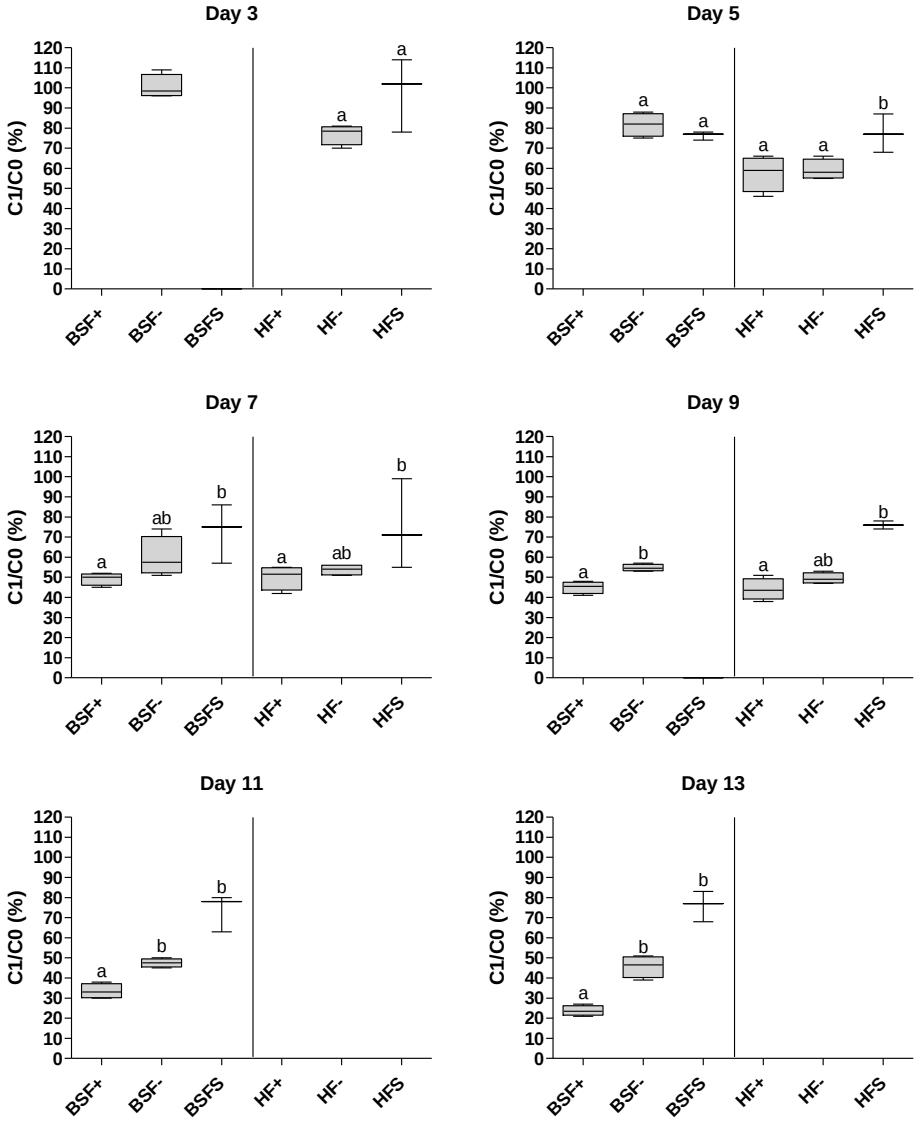

Figure S4

Whiskers plot: min to max. Effect of treatment on enzymatic and non-enzymatic degradation of AFB_1 (Kruskal-Wallis, pairwise comparisons; P = < 0.05). Significance was calculated within experiments (BSF or HF) only, and was done by comparing the treatments on each day (n=4: BSFL+, BSFL-, HF+, HF-; n=3: BSFS, HFS). Treatments with different letters differ significantly.

Chapter 6

Effects of aflatoxin B_1 on metabolism- and immunity-related gene expression in *Hermetia illucens* L. (Diptera: Stratiomyidae)

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Submitted

Abstract

Contamination of food products with mycotoxins such as aflatoxin B_1 (AFB_1) poses a serious risk to human health. Larvae of the black soldier fly (BSFL), *Hermetia illucens* (Diptera: Stratiomyidae) are capable of successfully metabolizing AFB_1 without any negative consequences on their survival or growth. Organic waste streams contaminated with mycotoxins can be upcycled into protein-rich BSFL as alternative feed for livestock and the left-over feed residue into nutrient-rich crop fertilizers. However, the underlying mechanisms that allow BSFL to metabolize AFB_1 have not been unravelled in detail. To elucidate these mechanisms, five-day-old BSFL were fed with a control or AFB_1-spiked diet. Larval samples were collected and subjected to RNA-Seq analysis to determine gene expression patterns. Provision of AFB_1-spiked diet resulted in up-regulation of 357 and down-regulation of 929 unique genes. Upregulated genes include multiple genes that are known to be involved in AFB_1 metabolism in other (insect) species. Downregulated genes were generally involved in growth, development and immunity of the insects. Overall, BSFL possesses a diverse genetic arsenal that encodes for enzymes capable of metabolizing AFB_1 without trade-offs for larval survival and growth. Negative effects of AFB_1 exposure on immunity-related processes are observed in the transcriptomic response indicative of a trade-off between detoxification and immune responses.

Introduction

Contamination of feed stuff with mycotoxins, and especially aflatoxin B_1 (AFB_1) is a global problem affecting food security and public health (Adeyeye *et al.* 2022; Meijer *et al.* 2021b). Innovative strategies to eliminate AFB_1 from the food chain are required and the use of detritivorous insects such as black soldier fly larvae (BSFL, *Hermetia illucens* L.) (Diptera: Stratiomyidae) has been proposed as a potential solution (*Chapter 2*; Niermans *et al.* 2021). The larvae of BSF have received increasing attention as potential source of alternative protein for livestock feed (Van Huis 2013; Van Huis 2016). The larvae are capable of feeding on a wide range of organic side streams, extracting and concentrating nutrients from nutrient-poor diets (Oonincx *et al.* 2015). The larvae have a high content of protein and other nutrients and are used as inputs for livestock feed (Dörper *et al.* 2021), while the by-products like frass and exuviae show potential as organic amendments (Barragán-Fonseca *et al.* 2022).

The potential of BSFL to utilize a wide variety of organic side-streams including contaminated commodities like maize, peanuts, etc. has previously been investigated (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 7*; *Chapter 8*; Gold *et al.* 2023; Leni *et al.* 2019; Meijer *et al.* 2019; Niermans *et al.* 2023c; Purschke *et al.* 2017). Concentrations of AFB_1 fed to BSFL varied between 8 and 500 µg/kg feed and none of these studies recorded any negative effect on larval survival or growth (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 7*; Meijer *et al.* 2019; Niermans *et al.* 2023c). Interestingly, all these studies showed that BSFL did not accumulate AFB_1 within their

body. Furthermore, based on the molar mass balance that quantifies which proportion of AFB_1 in the substrate is recovered in larvae and the mixture of feed substrate residue and larval excreta showed that a large fraction of AFB_1 remained unaccounted for (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Niermans *et al.* 2023b). There is a distinct possibility that the larvae metabolized AFB_1 into 'novel' unknown metabolites, which therefore could not be included in the mass balance. In particular, the biochemical pathways that allow BSFL to metabolize AFB_1 requires further understanding.

Molecular mechanisms involved in AFB_1 metabolism have not been thoroughly investigated in insects in contrast to studies on human or mammalian models. However, it is known that insects possess cytochrome P450s (Berenbaum *et al.* 2021; Berenbaum and Johnson 2015; Niu *et al.* 2008; Zeng *et al.* 2006), glucosyltransferases (De Zutter *et al.* 2016; Pan *et al.* 2020) and other metabolic conjugation systems (Wilkinson 1986) that could potentially be involved in AFB_1 metabolism. More specifically, the genome of BSF contains significant expansion in CYP450 gene families which are associated with xenobiotic detoxification (Zhan *et al.* 2020). Furthermore, in BSFL CYP450s are responsible for the metabolism of AFB_1 into aflatoxin P_1 (AFP_1) and a cytoplasmic NADPH-dependent reductase for the transformation of AFB_1 into aflatoxicol (AFL) (Meijer *et al.* 2019). Here, we aim to investigate the biochemical pathways involved in AFB_1 metabolism.

Based on the existing data, AFB_1 exposure does not result in lethal consequences or growth reduction in BSFL (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Meijer *et al.* 2019). This may be the result of AFB_1 - mediated induction of AFB_1 metabolism in BSFL. However, metabolic induction often results in negative consequences on growth and survival of the insect; as observed in *Helicoverpa armigera* (Hubner) (Elzaki *et al.* 2019). AFB_1 feeding leads to induction of *CYP6AE19* in *H. armigera*, which in turn results in bioactivation of AFB_1 and increases larval mortality (Elzaki *et al.* 2019). However, consequences of AFB_1 feeding on transcriptomic response of BSFL are not yet known. In the current study, five-day-old BSFL were fed on a control diet or on an AFB_1-spiked diet after which whole-larvae RNA samples were collected at three time points (6, 24 and 72 h). The larval response to AFB_1 exposure was evaluated using RNA-sequencing with the aim of evaluating AFB_1 - mediated effects on AFB_1 detoxification, metabolism and immune functions in BSFL.

Materials and Methods

Insect rearing

The insects used in the experiments were derived from a colony of *Hermetia illucens* L. (Diptera: Stratiomyidae) maintained at the Laboratory of Entomology, Wageningen University (the Netherlands) in a climate room (27 ± 1°C, 70 ± 10% R.H., L12 : D12). Eggs laid within a period of six hours were collected from this colony. Two random egg clutches (a total of ~1500 eggs) were

placed on a chickenfeed diet (150 g + 300 mL water) (Kuikenopfokmeel 1; Kasper Faunafood, Woerden, the Netherlands) in a plastic container (15.5 × 10.5 × 6 cm). The container was closed with a plastic lid with a rectangular hole (7 x 5.5 cm) that was covered with nylon mesh to facilitate ventilation.

Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1); from Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and from TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

Spiked feed preparation

BSFL were fed on either a control diet or a diet spiked with 20 µg/kg AFB_1. This concentration was based on the maximum level (ML) of AFB_1 allowed in any feed material in the EU, set in Directive 2002/32/EC (EC 2002a). Both diet treatments were performed in four replicates. To prepare the spiked diet, 1.9 mL of the AFB_1 solution (suspended in MeOH; final percentage of 0.2% MeOH in the diet) was mixed with 618 mL water and 333 g chickenfeed (moisture content 65%; final percentage of 0.2% MeOH in the diet). The control chickenfeed diet was prepared in the same way, with addition of 1.9 mL of MeOH leading to a final percentage of 0.2% MeOH in the diet. Both control and AFB_1-spiked diets were mixed manually for 15 min. An overview of the intended and the final experimentally determined concentrations in the diets is presented in Table 1.

In order to determine homogeneity of the spiked feed, eight samples were taken from the AFB_1 diet. Homogeneity was confirmed when the measured concentrations (relative standard deviation of the replicates) in the samples differed ≤ 20% from each other. Four samples taken from the control diets were analysed to check whether aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFL, AFP_1, AFM_1 and AFQ_1) were absent in these diets.

Feed transfer

Five-day-old BSFL (5DOL) were taken randomly and divided over groups of 150 larvae and weighed using a NewClassic MF ML54 (Mettler Toledo) balance to determine the average biomass at the start of the experiment (22.41 ± 0.19 mg per larva). The larvae were transferred to 100 g of diet for the respective treatments: control and AFB_1-spiked diet (20 µg/kg AFB_1). Four containers (480 mL rearing cups; BugDorm insect pots purchased from MegaView Science Co. Ltd., Taichung, Taiwan) were set up per diet type, and placed in a climate room (27 ± 1°C, 70 ± 10% R.H., L12:D12).

Sample collection and RNA isolation

Larval samples for RNA-Seq analyses (four times two larvae from each container, resulting in four biological replicates) were collected from control and AFB_1-spiked diets respectively at three timepoints (6 h, 48 h and 72 h). The remainder of the larvae were allowed to continuously feed on the AFB_1-spiked substrate for seven days. On day seven after the start of the feeding trial, the 12-day-old BSFL were separated from the substrate, washed, dried, counted and weighed. Samples of the residual material (mixture of left-over substrate and larval excreta, called frass) were collected to analyse aflatoxin concentrations. Samples for RNA-Seq analysis were collected at multiple timepoints during the feeding trial to account for biological processes affected in the larvae due to AFB_1 feeding, irrespective of chronology. The larvae were weighed and cleaned in distilled water twice for 5 s and air-dried on a clean paper towel. Total RNA was extracted and DNase treatment performed as described Shah *et al.* (2023). The quality and quantity of extracted RNA was assessed using a DS-11 series spectrophotometer (DeNovix, USA). The RNA integrity of the samples was monitored on 1% agarose gel and further assessed with Agilent 6000 Bioanalyser (Agilent Technologies, Santa Clara, CA, United States). RNA was stored at -80°C until further use.

Library preparation and RNASeq

cDNA libraries were constructed using TruSeq Stranded Total RNA Library Preparation (Illumina, San Diego, CA, United States) with input of 2.5 µg total RNA per sample. The cDNA libraries were sequenced (150 bp, paired-ended) on an Illumina NovaSeq6000 instrument. Sequencing was performed to an average depth of 19.3 M reads per sample. FASTQ sequence files were generated using bcl2fastq2 version 2.20, which included Illumina-Chastity quality filtering with default settings. Subsequently, reads containing adapters and/or PhiX control signal were removed using an in-house filtering protocol (BaseClear B.V., Leiden). In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 99 bp). Second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8 (Andrews 2010) with a Q-score of >35. Read quality was assessed using multiQC (Ewels *et al.* 2016). Reads were processed with Cutadapt (Martin 2011) to remove any remaining sequencing adaptors with the following settings: -m (minimum length) 100 -q (phred) 30 --no-indels.

Sequence alignment and gene counts

The FASTQ files were aligned to the reference genome of *H. illucens* (RefSeq assembly accession: GCF_905115235.1) using STAR (Dobin *et al.* 2012), and genes were annotated using the gene annotation file (gtf) (Generalovic *et al.* 2021) from the NCBI database. The FASTQ sequences were aligned to the genome in --quantMode to obtain read counts per gene directly. On average,

91.2% of the reads (average GC% = 39%) mapped to the reference genome (Table S1). Raw sequencing data will be available.

Principal component analysis (PCA) and analysis of Differentially Expressed Genes (DEG)

The differences in sample library sizes were determined using estimateSizeFactors and estimateDispersions (negative binomial distribution) functions of the DESeq2 package (Love *et al.* 2014). Further, the count data were transformed to $\log_2$ scale to minimize differences between samples for rows with small counts and normalized with respect to library size using rlogcounts function of DESeq2 package (Love *et al.* 2014). Principal component analysis (PCA) was performed on the top 500 genes with maximum variance across samples with PCAexplorer (Marini and Binder 2019). rlogcounts were used as input for generating the PCA plot and the genes in the top and bottom loadings for each of the PC axes were extracted and functionally characterized. The gene read counts obtained via STAR were processed in R using the DESeq2 package (Love *et al.* 2014). A DESEq matrix (design = ~ treatment + time) was created to determine differentially expressed genes (DEGs). Genes with low count numbers (= < 20) across all samples were filtered out. The list of Differentially Expressed Genes (DEGs) was extracted by drawing contrasts between AFB_1 and control samples for the three time points by using the *results* function from the DESeqDataSet. Genes were identified as DEGs when their false discovery rate (FDR) was lower than 0.001 and $\log_2$ fold change (LFC) was higher than 2 (*i.e.* four time higher expression) in treatment than control samples.

Gene Ontology and Pathway analysis

Identified DEGs were used as input for Gene Ontology (GO) enrichment analysis to determine the affected processes. To explore the structure in gene expression, relative counts of DEGs were plotted and further segregated into different clusters (hierarchical clustering), whose functions in biological processes were determined using the topGO package (Alexa and Rahnenfuhrer 2022).

Aflatoxin analysis

The extraction procedure for the substrate and residual material was performed as described by Camenzuli *et al.* (2018). This method is an in-house method at Wageningen Food Safety Research which is validated according to SANTE/12682/2019 (EC 2019b). Extractions from the larval material were performed according to a slightly adjusted protocol. In short, 1 g of larval material and 3 mL MilliQ water were mixed. After 15 min, 4 mL of extraction solvent (acetonitrile/acetic acid 99:1 (*v/v*)) was added and the mixture was shaken in a head-over-head shaker for 30 min. After addition of 1.6 g of magnesium sulphate the samples were mixed manually, vortexed and centrifuged (MSE Falcon 6-30) for 10 min at 3000 rpm. Aflatoxin concentrations in both the

substrate and larval material were quantified by means of standard addition, therefore, each extract was prepared with and without the addition of a standard mix containing eight aflatoxins (30 µg/kg AFB_1, 5 µg/kg AFB_2, AFG_1, AFG_2, AFL, AFP_1, AFM_1 and AFQ_1). 200 µL sample extract was mixed with either 190 µL water and 10 µL of the standard mix or only 200 µL water in a syringeless PTFE filter file (Mini-UniPrep, Whatman, Marlborough, MA). The vials were capped, vortexed and placed in the refrigerator for 30 min after which they were closed and stored at 4°C until LC-MS/MS analysis.

The LC-MS/MS system consisted of a Waters Acquity injection and pump system (Waters, Milford, MA) and an AB Sciex QTRAP 6500 triple quad system equipped with an electrospray ionization source. LC-MS/MS analyses were performed exactly as described in *Chapter 4* (Niermans *et al.* 2023b) and in the current experiment aflatoxin analyses were also solely performed in positive ESI mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Table S2). LC separation was performed with an Acquity UPLC HSS T3 1.8 µm 100x2.1 mm column (Waters, Milford, MA).

Data analysis

All statistical analyses and image/plot generation were performed in RStudio (R Core Team 2023). Data collected on the survival and biomass of the larvae at harvest were used to determine the effect of aflatoxin exposure on BSFL. Differences in larval survival and biomass were determined with Kruskal-Wallis test; α was set at 0.05. LC-MS/MS data was analysed with SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA). Peak identification was done according to SANTE/12682/2019 (EC 2019b). Concentrations were calculated by dividing the peak area of the sample by the area of the sample with standard addition minus the area of the sample and multiplied by the addition level of the respective aflatoxin. Quantification of concentrations was corrected for recovery percentages of the included aflatoxins (Table S3), and was done following Regulation (EC) 401/2006 (EC 2006a). Limits of detection (LOD) and limit of quantification (LOQ) for each of the aflatoxins in their respective matrices were determined (Table S4) as described in *Chapter 4* (Niermans *et al.* 2023b). Aflatoxin concentrations below the LOQ were set to zero.

Genes known to be involved in a) metabolism of AFB_1 in other organism models, and b) development, biomass and immunity of BSFL were assessed separately. Based on scientific evidence from other organisms and observations made in this study, potential pathways that allow BSFL to successfully metabolize aflatoxins are discussed.

Results

Aflatoxin analysis, larval survival and biomass

Aflatoxin concentration in the AFB_1 diet was 19.7 ± 1.0 µg/kg, the deviation range being within ± 20%, thereby confirming the homogeneous distribution of the spiked substances in the substrate. No mycotoxins were detected in the control diets (Table 1, Table S5). The residual material, collected after seven days of exposure, contained AFB_1 (35.42 ± 3.79 µg/kg) and AFP_1 (9.69 ± 1.40 µg/kg).

Table 1. Overview of experimental diets

#	Diet	Replicates	Intended mycotoxin concentration (µg/kg ww)	Actual mycotoxin concentration (µg/kg ww)[1]	Percentage of MeOH in diet
1	Control	4	0	< LOD	0.2%
2	Spiked with AFB_1	4	20	19.71 ± 1.02	0.2%

[1] Average concentration in the replicates (n=4 per diet treatment, each replicate was a group of 150 larvae) as determined by LC-MS/MS analysis. Individual measurements for the replicates can be found in Table S1. ww: wet weight; < LOD: below limit of detection.

Survival of BSFL feeding on either control or AFB_1-spiked diet over a period of seven days was high (88-94%, Table S6) and not affected by AFB_1 exposure (Kruskal-Wallis test, $p > 0.05$; Figure 1a). The biomass of the BSFL collected for RNA-Seq analyses was not affected by AFB_1 exposure after 6 h, 48 h and 72 h (Table S7) or in 12DOL at the end of experiment (Kruskal-Wallis test, $p > 0.05$; Figure 1b).

Sequence quality and Principal Component Analysis (PCA)

Average library size was 17.2 million reads per sample (39% GC content), of which 91.2% reads (~ 15.7 million reads) were mapped to the annotated genome of *H. illucens* (Table S1). A total of 15,196 genes were found with a gene count in the mapped dataset. Filtering out genes with counts less than 20 copies resulted in a dataset with a total of 11,429 genes.

Multivariate analysis revealed distinct separation between diet treatments (Figure 2). Although two data points overlap, PC1 clearly segregates the samples between the two treatments (*i.e.* control and AFB_1) irrespective of sampling time. Together, PC1 and PC2 account for 76% of the variation.

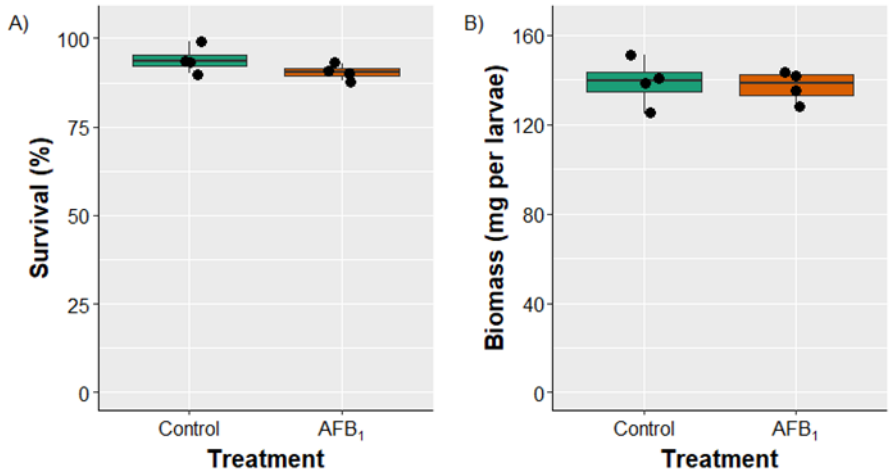

Figure 1. Boxplot of survival (A) and biomass (B) of BSFL reared on control and AFB_1-spiked diets for a period of seven days (5 days old larvae till 12 days old). No significant differences were observed for BSFL survival and biomass when fed on control or AFB_1 diets (Kruskal-Wallis test; $p > 0.05$). Data on survival and weight biomass per replicate after seven days of exposure are presented in Table S6.

The genes contributing to separation between the treatments in PC1 were identified and characterized (Figure S1). Top loadings (*i.e.* genes contributing to positive values on PC1, *i.e.* AFB_1 effects on BSFL gene expression) of PC1 included cytochrome P450 6a14 (CYP6a14, LOC119646754), cytochrome P450 6g2 (CYP6g2, LOC119655376) and enzyme-like aldo-keto reductase family 1 member B1 (LOC119649999). Bottom loadings of PC1 (*i.e.* genes contributing to negative values on PC1, corresponding to gene expression of control larvae) contained genes involved in the production of structural proteins (*e.g.* endocuticle structural glycoprotein SgAbd-2, pupal cuticle protein and glycine-rich structural protein) in the larvae (Silvert *et al.* 1984; Ye *et al.* 2021).

Analysis of Differentially Expressed Genes (DEGs)

A total of 1277 genes were differentially expressed ($|\log_2 FC| \geq 2$ and false discovery rate < 0.001) between larvae fed on AFB_1-contaminated and control diet over the three tested timepoints, corresponding to 11% of the total number of genes found within the dataset. A total of 357 genes were uniquely upregulated while 929 genes were uniquely downregulated as a result of AFB_1 feeding across all timepoints. Interestingly, more genes were distinctly downregulated at each timepoint compared to those that were upregulated (Figure 3). Some genes are affected (significantly up- or down-regulated, *i.e.* log2fold change) across two consecutive sampling timepoints as a result of AFB_1 feeding (Table S8 and S9).

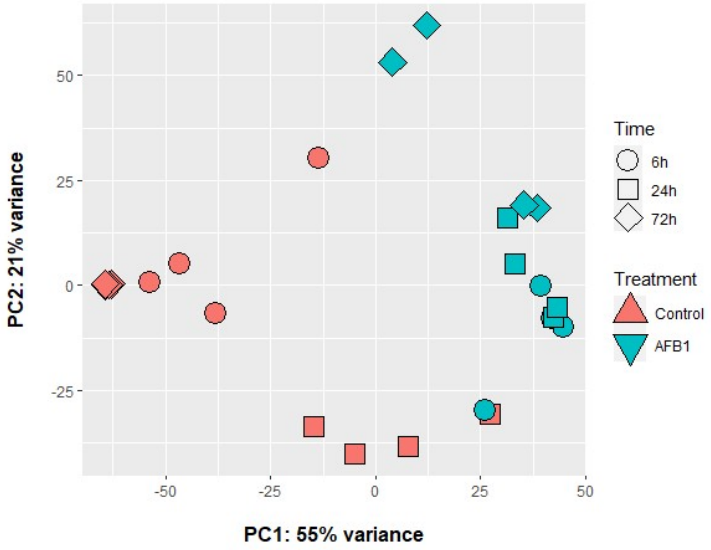

Figure 2. Principal Component Analysis of transcriptome of BSFL fed on control diet (chickenfeed) and AFB_1-spiked diet (chickenfeed with 20 µg/kg AFB_1). Different sampling timepoints are indicated as different shapes and treatments as colours (orange = control, blue = AFB_1 fed larvae).

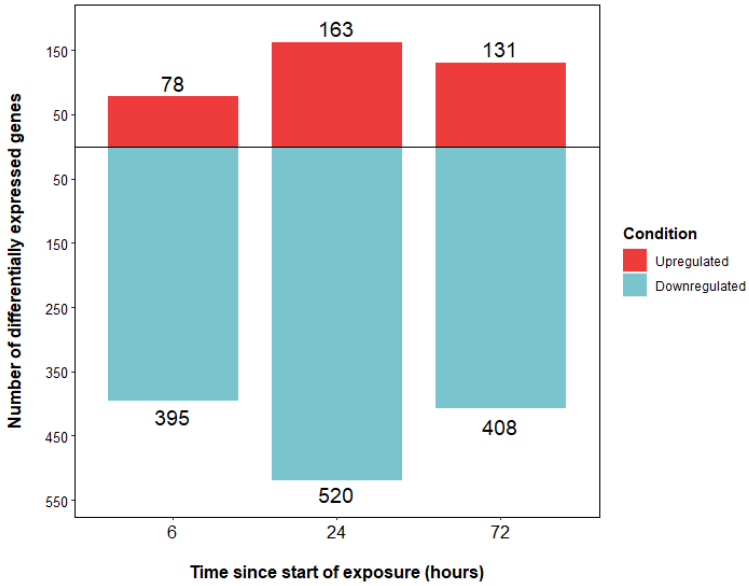

Figure 3. Number of differentially expressed genes (DEGs); either upregulated (in red) or down-regulated (in blue) in BSFL as a result of continuous feeding on a diet contaminated with AFB_1 compared to a control diet, sampled at three timepoints.

Gene ontology (GO)

GO enrichment analysis of DEGs indicates enrichment of genes encoding various enzymes *e.g*. methyltransferases, oxidoreductases, carboxypeptidases, exopeptidases (Figure 4a). Conversely, DEGs involved in processes in biological processes like proteolysis, primary lipid and carbohydrate metabolic processes; together with molecular functions (*i.e*. such as lipase and hydrolase activity, lipid transport, phospholipase, peptidase and carbohydrate derivative functions) were downregulated as a result of feeding on an AFB_1-contaminated diet (Figure 4b).

To categorize the functions of genes of which the expression was affected by feeding on an AFB_1-contaminated substrate, DEGs were divided into 9 clusters based on hierarchical clustering (Figure S2). Upon division of DEGs into separate clusters, relative expression of DEGs was plotted in a heatmap (Figure 5).

Genes belonging to Clusters 1 – 5 were downregulated upon AFB_1 feeding, with the exception of genes involved in Cluster 3 which were upregulated at 72 h. The genes within these clusters are known to be involved in growth and development processes in insects. Genes such as those encoding for trypsin and ctenidin, were included in Cluster 1 and are involved in multiple processes such as rRNA processing, ribosome biosynthesis, thymine and uracil catabolic processes, peptide crosslinking and DNA-templated transcription processes. Genes included in Cluster 2 encode for *e.g.* lipase, ecdysteroid-regulated 16 kDa protein, serine protease 7, β-trypsin, cecropin (LOC119657589 and LOC119657650) and chymotrypsin were downregulated across all three timepoints as a result of AFB_1 feeding. The genes within this cluster are known to contribute to antibacterial humoral response, carbohydrate phosphorylation, galactose and lipid metabolic processes. Similarly, genes encoding for phenoloxidase, mucin, neurotrophin, and titin were grouped in Cluster 3. The downregulated genes within Cluster 3 are involved in proteolysis and response to oxidative stress. Genes in Cluster 4 were significantly downregulated across all three timepoints and included genes encoding *e.g.* spaetzle protein, odorant-binding proteins, multiple cuticle proteins, peroxidases, esterase B1, dual oxidase, protein takeout. These genes play roles in the response to oxidative stress, lipid metabolism, proteolysis, arachidonic acid secretion, and melanin biosynthesis and were significantly downregulated upon exposure to AFB_1. Genes in Cluster 5 were also significantly downregulated as a result of AFB_1 feeding and included genes encoding for glycine-rich protein DOT1, phenoloxidase 3, cadherin-23, mantle protein, myosin-11, lipase-3, neprilysin-4, Toll-receptor 6. The processes that were negatively affected included DNA-templated transcription, homophilic cell adhesion via plasma membrane adhesion molecules, regulation of signalling and carbohydrate metabolic process.

Genes in Clusters 6 – 9 were significantly upregulated as a result of AFB_1 feeding and are known to be involved in xenobiotic metabolism, detoxification and insecticide resistance. Cluster 6 contains genes encoding for *e.g.* cytochrome b5 and phosphomevalonate kinase. These genes

are involved in processes such as cholesterol biosynthesis, histone H3-K79 methylation and protein import into peroxisome matrix. Genes in Cluster 7 encode for multiple enzymes involved in xenobiotic metabolism such as CYP6a14, glutathione S-transferase 1, alcohol dehydrogenase 1, aldo-keto reductase family 1 member B1, UDP-glucosyltransferase 2, farnesol dehydrogenase, esterase E4, sorbitol dehydrogenase, and glycine N-methyltransferase. These genes contribute to biological processes such as arginine biosynthetic process via ornithine, initiation of translation and mitochondrial electron transport. Genes within Cluster 8 were distinctly upregulated at 72 h post AFB_1 feeding, and included genes encoding for esterase E4, CYP450 6a13, CYP450 12a5, kynurenine formamidase, chymotrypsin-C, glucose dehydrogenase, CYP450 4d14, β-thymosin, CYP450 307a1, carboxypeptidase B, CYP450 12A2. The processes affected due to upregulation of these genes are proteolysis, sulfate transport, cholesterol and carbohydrate metabolism, transmembrane transport and inositol catabolism.

Genes in Cluster 9 encode glutathione S-transferase, zinc carboxypeptidase and CYP450 12A2. Upregulation of these genes stimulates ubiquitin protein ligase activity, protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway and regulation of actin cytoskeleton organization.

The majority of upregulated genes of interest are linked to xenobiotic metabolism (*e.g*. CYP6a14, CYP6a13, CYP4d14, farnesol dehydrogenase, cytochrome b5). The downregulated genes of interest are mainly associated to processes involved in development (*e.g*. endocuticle glycoprotein SgAbd-2, larval cuticle proteins) and immunity (*e.g*. glycine-rich protein DOT1) of BSFL. Gene families known to be involved in AFB_1 detoxification in mammalian model organisms selected are also differentially expressed in BSFL (Figure 6a). Exposure through feeding on an AFB_1-spiked diet resulted in distinct upregulation of various genes from the cytochrome b, cytochrome C and CYP450 families. Furthermore, genes encoding for various dehydrogenases, transferases, peptidases, but also juvenile hormone epoxide hydrolase, aldo-keto reductase B1, and esterase E4 were clearly upregulated upon AFB_1 exposure. Furthermore, genes known to be involved in growth, development and immunity were selected from the DEG list (either upregulated or downregulated at at least one sampling timepoint) and plotted to explore their expression over the sampling timepoints (Figure 6b). Genes coding for cuticle formation proteins were significantly downregulated. Similarly, multiple genes related to insect immunity such as cecropin, ctenidin, phenoloxidase and gut homeostasis such as dual oxidase were significantly downregulated upon feeding on an AFB_1-spiked diet. Interestingly, genes encoding for enzymes involved in intermediary metabolism and proteolytic activities like chymotrypsin, lipase and trypsin were significantly downregulated at the early timepoint upon exposure to AFB_1 feeding. However, chymotrypsin and glycine-rich protein DOT1 were significantly upregulated at 72 h upon exposure to AFB_1.

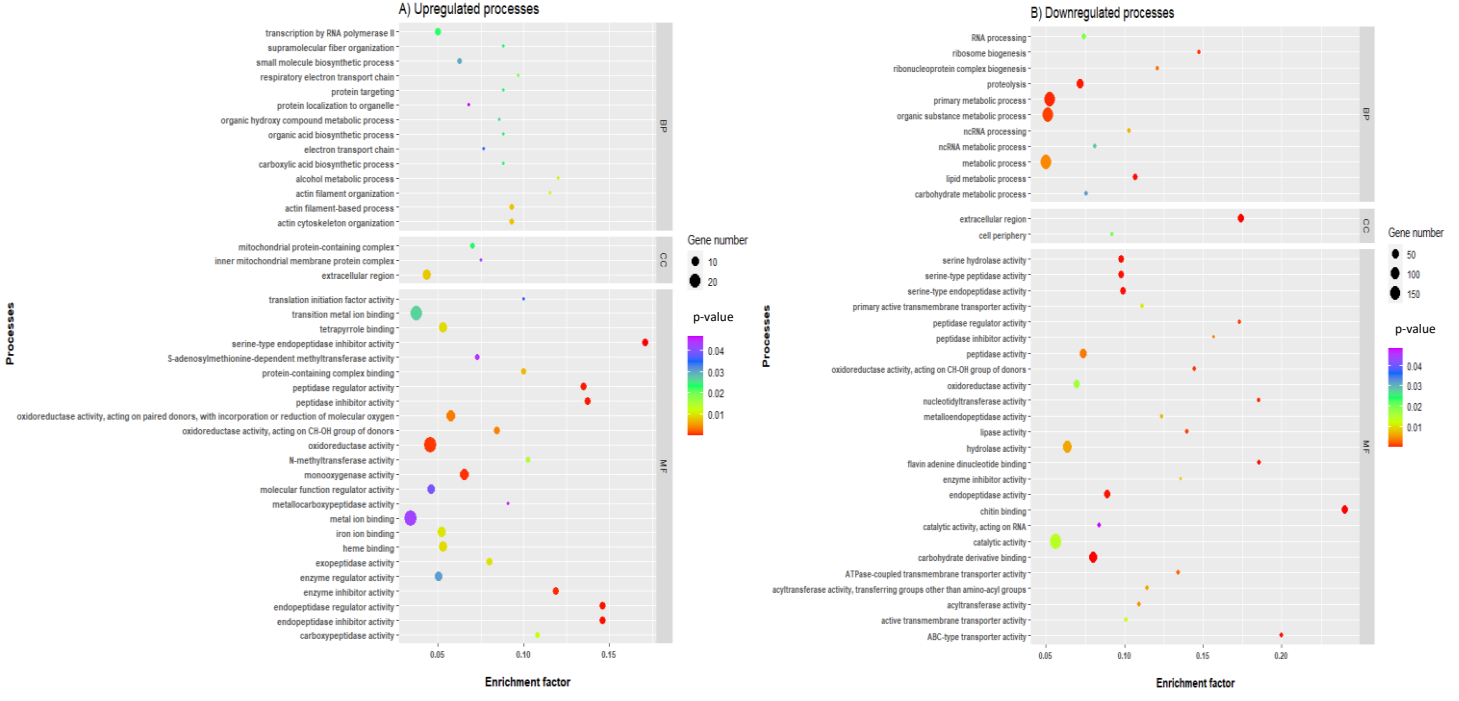

Figure 4. Overview of GO analysis outcomes in terms of processes that are (A) upregulated (from genes with LFC ≥ 2 and FDR ≤ 0.001), and (B) downregulated (from genes with LFC ≤ -2 and FDR ≤ 0.001) in BSFL as a result of AFB_1 feeding compared to control diet. Affected processes have been classified into three categories: biological processes, cellular components and molecular functions. Enrichment factor indicates the number of significantly affected genes compared to the total number of annotated genes involved in a particular process/function. Size of the dot indicates the number of genes involved in the GO process and the colour indicates the associated p-value (red = highly significant).

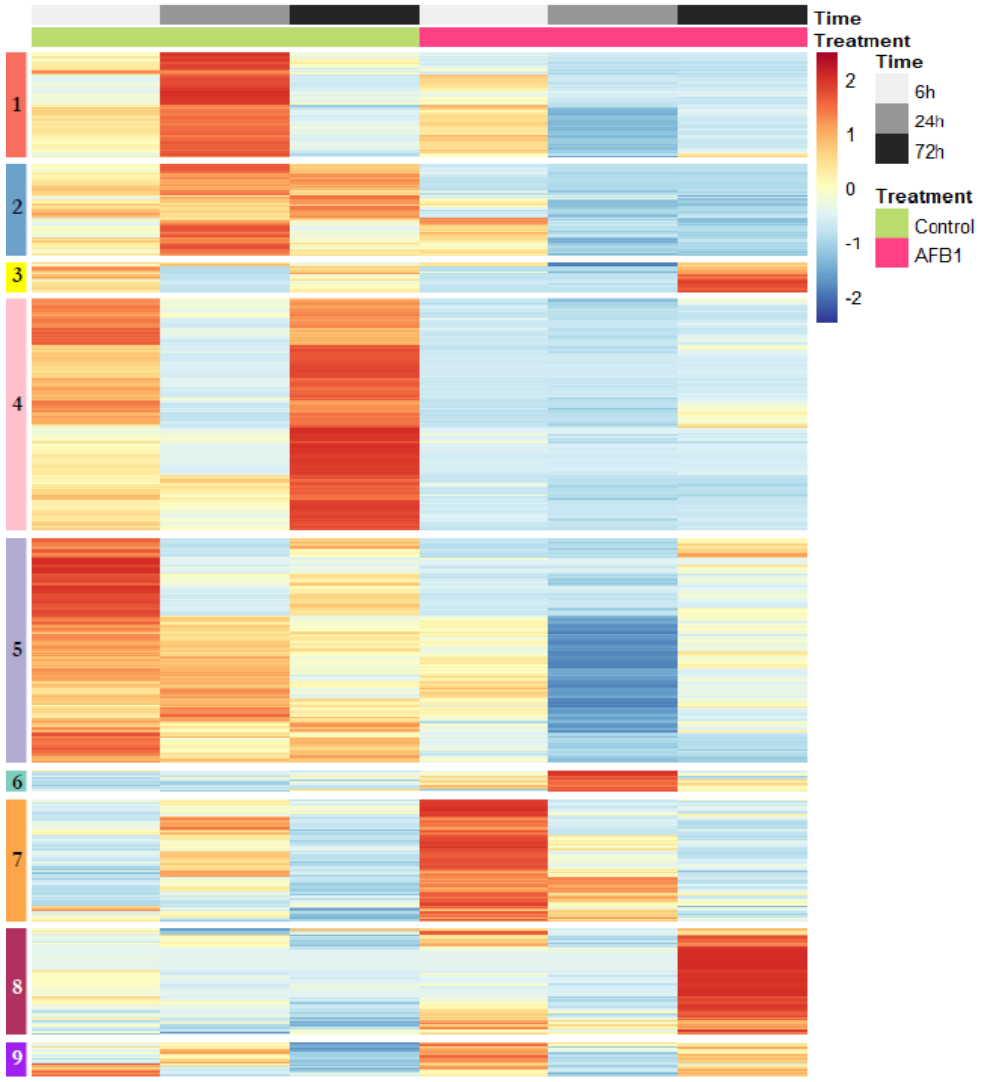

Figure 5. Heatmap indicating relative expression of differentially expressed genes (DEGs) in BSFL at three timepoints after the start of feeding on a control or AFB_1-spiked diet. DEGs were grouped into nine distinct clusters based on their expression patterns across time and treatment combinations. Upregulated genes are shown in red while downregulated genes are shown in blue.

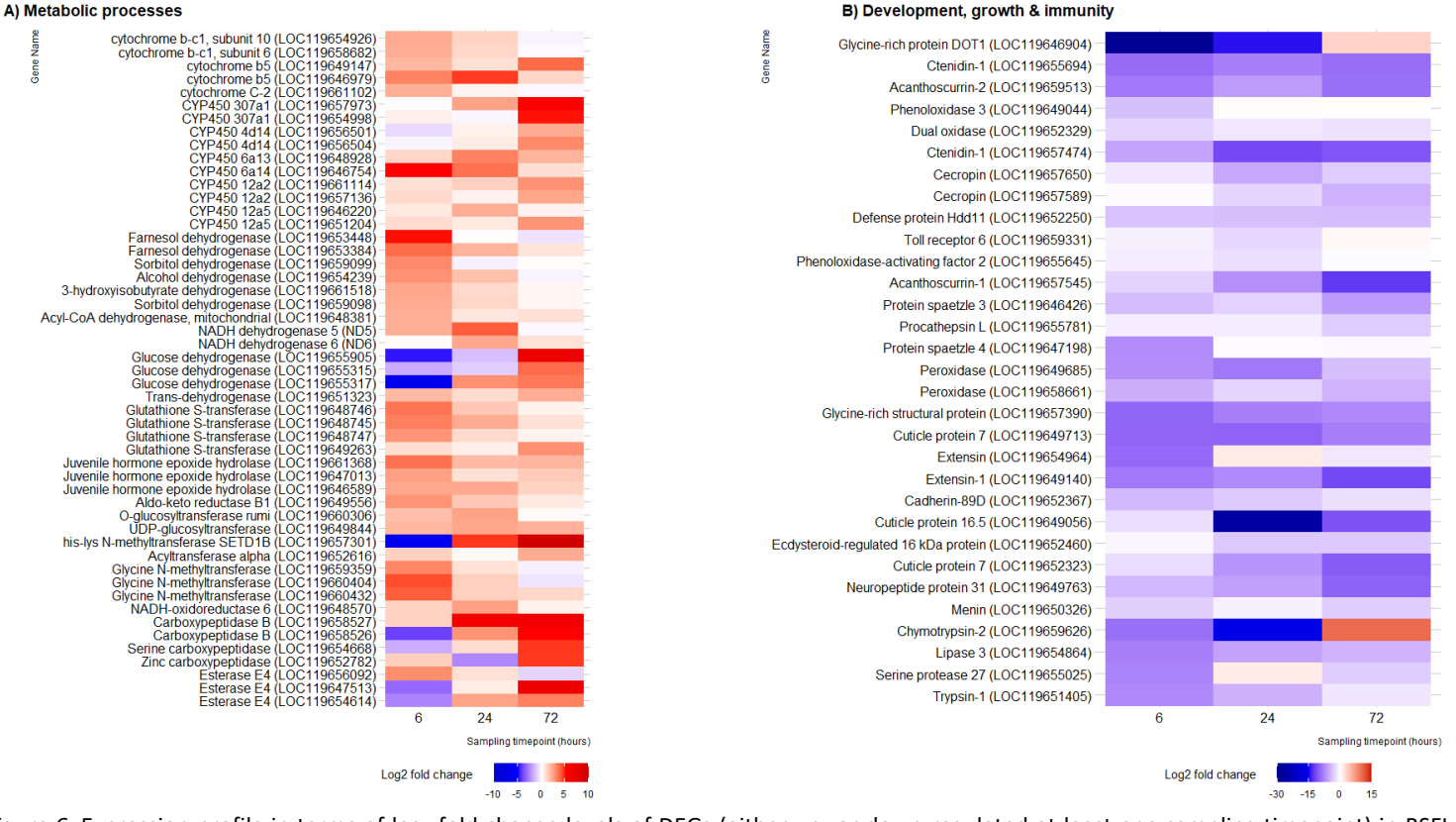

Figure 6. Expression profile in terms of $\log_2$-fold change levels of DEGs (either up- or down-regulated at least one sampling timepoint) in BSFL affected by AFB_1 exposure through feeding compared to control diet. These genes encode for different enzymes involved in A) xenobiotic metabolism of AFB_1 (gene selection based on mammalian model organisms), and B) different developmental, growth and immunity related pathways.

Discussion

Feeding five-day-old BSFL on an AFB_1-spiked diet did not affect their survival and biomass in comparison to larvae fed on a control diet over a period of seven days. Previous AFB_1 feeding experiments on BSFL found similar results (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; *Chapter 7*; Meijer *et al.* 2019; Niermans *et al*. (2023b); Niermans *et al.* 2023c). Analysis of the larval transcriptome indicates a significant upregulation of genes associated with xenobiotic metabolism when fed on a diet spiked with 20 µg/kg AFB_1. In contrast, multiple genes involved in growth, development and immunity were significantly downregulated suggesting a trade-off between detoxification and these latter processes. BSFL possess a considerable number of genes encoding for enzymes known from other insect species to be involved in xenobiotic transformation. Xenobiotic transformation entails processes facilitating excretion of endogenous and exogenous substances *e.g.* secondary plant metabolites, insecticides or mycotoxins, among others (Almazroo *et al.* 2017). These substances are altered via a series of reactions transforming the xenobiotic into more polar water-soluble metabolites, which can be excreted. In general, xenobiotic elimination occurs in three phases, *i.e.* Phase I metabolism, Phase II metabolism, and Phase III (excretion) (Esteves *et al.* 2021). The reactions involved in these phases may occur either sequentially or simultaneously (Meyer 1996).

Phase I

Phase I metabolism mainly results in conversion of xenobiotic compounds into more polar/water- soluble metabolite(s). Phase I metabolites are often reactive, and can become substrates for Phase II metabolism (Meyer 1996). Reactions involved in Phase I metabolism include oxidation, hydrolysis and reduction. Cytochrome P450s and aldo-keto reductases are enzymes known to be involved in a variety of (xenobiotic) metabolic pathways and play a role in resistance to xenobiotics in insects (Feyereisen 2006). In insects (*e.g*. *Drosophila melanogaster, Trichoplusia ni* and *Helicoverpa armigera*), CYPs are known to play a role in the detoxification of aflatoxins (Berenbaum *et al.* 2021). The CYP family also appears to play an important role in the current study as CYP6a14 and CYP6g2 were among the main genes contributing to separation between the treatments. In the current study, CYP6a14 and CYP6g2 were upregulated after 6 h of feeding on AFB_1. Upregulation of CYP6a14 was also shown after *D. melanogaster* larvae were fed a sterigmatocystin-contaminated diet (Trienens *et al.* 2017). Sterigmatocystin shares its biosynthetic pathway with the aflatoxins and is a known precursor of AFB_1 (Zingales *et al.* 2020). Furthermore, CYP6a14-1 as well as esterases contributed to insecticide resistance in the aphids *Sitobion avenae* and *Myzus persicae* (Field and Devonshire 1998; Li *et al.* 2007; Zhang *et al.* 2020). As opposed to CYP6a14, the P450s CYP4d14 and CYP21a2 were upregulated after 72 h of feeding on an AFB_1-contaminated diet. CYP4d14 as well as CYP6g2 have monooxygenase, oxidoreductase, and electron transporter activity, amongst others (Kalajdzic *et al.* 2012; Sun *et al.* 2006), and may be involved in the metabolism of insect hormones and breakdown of

synthetic insecticides. The product of the CYP21a2 gene generally seems to be involved in hormone synthesis (Eachus *et al.* 2017). Multiple studies in which BSFL were fed an AFB_1-contaminated diet, including the current study, showed formation of the AFB_1 metabolite AFP_1 in the residual material (*Chapter 4*; *Chapter 7*; *Chapter 8*; Gold *et al.* 2023; Niermans *et al.* 2023b; Niermans *et al.* 2023c). As CYP450s are responsible for the metabolism of AFB_1 into AFP_1 (Meijer *et al.* 2019), one or multiple of the previously mentioned CYPs were likely involved in this transformation. In addition to the gene expression of CYP450 enzymes, cytochrome b5 (cyb5) was upregulated at 24 h and 72 h. Cyb5 participates in the oxidation of xenobiotics (Hildebrandt and Estabrook 1971), and stimulated the activation of AFB_1 to a greater extent when it was added together with CYP1a, 1c or 1d, as compared to AFB_1 activation caused by the CYPs alone (Ueno *et al.* 1983).

Based on the known metabolic pathways of AFB_1 (Figure 7) and the information presented so far, it seems likely that BSFL are able to transform AFB_1 via CYP enzymes and/or cyb5 into an AFB_1-epoxide (AFB_1-*exo*-8,9-epoxide and/or AFB_1-*endo*-8,9-epoxides). This seems to be further supported by the high expression levels of juvenile hormone epoxide hydrolase 1 (*JHEH*) observed in this study. JHEH is involved in juvenile hormone degradation in insects, and is hypothesized to possess epoxide hydrolase activity (Anspaugh and Roe 2005; Sun *et al.* 2006). In mammals, epoxide hydrolases are known to convert epoxides into their corresponding dihydrodiols, via hydrolysis (Oesch *et al.* 1971), and are therefore considered key enzymes in the detoxification of genotoxic epoxides (Kelly *et al.* 2002). Furthermore, genes encoding aldo-keto reductase family 1 member B1 (*AKR1B1*), were among the main genes contributing to separation between the treatments (Figure 7) and were significantly upregulated after 6 h of feeding on an AFB_1-spiked diet. *AKR1B1* belongs to the aldo-keto reductases (AKRs), which are cytosolic NADP(H)-dependent oxidoreductases (Ellis *et al.* 1993; Knight *et al.* 1999). The aflatoxin B_1 aldehyde reductases, and specifically the NADPH-dependent aldo-keto reductases of rats (AKR7A1) and humans (AKR7A2), are known to catalyse the reduction of aflatoxin dialdehyde to its corresponding di-alcohol (Ellis *et al.* 1993; Khayami *et al.* 2020; Knight *et al.* 1999). During this transformation step, protein-binding abilities are eliminated and thereby represent an important detoxification route of AFB_1 (Cao *et al.* 2022). The significant upregulation of *JHEH* expression in the current study, might indicate formation of the AFB_1-dihydrodiol. Further conversion of AFB_1-dihydrodiol into the corresponding dialdehyde might have occurred (Figure 7) after which AKR1B1 may convert AFB_1-dialdehyde into AFB_1-dialcohol.

Phase II

Phase II metabolism involves conjugation of the xenobiotic or its metabolites (possibly formed during Phase I metabolism) to another endogenous hydrophilic group. Conjugation will result in the formation of a large inactive polar and more water soluble metabolite, which can be excreted by the Malpighian tubules (Dow and Davies 2006). Xenobiotics may be conjugated with amino

acids (glycine, taurine, glutamic acid), glucuronic acid, glutathione, methyl or acetyl groups or sulphate (Almazroo *et al.* 2017; Omiecinski *et al.* 2010). In the current study, upregulation of glutathione S-transferase 1 and UDP-glucosyltransferase 2 at 6 h and 72 h might indicate that Phase II metabolism occurred. UDP-glycosyltransferases (UGT) are a superfamily of enzymes that catalyse glucosidation. As insects are known to metabolize certain xenobiotics via glucosyltransferases (Bock 2016), it is hypothesised that AFB_1-glucose conjugates could be formed by insects (Gützkow *et al.* 2021). Whether AFB_1-dialcohol serves as a binding substrate for glucose conjugation is thus far unknown. More extensive analysis (*e.g*. the addition of a glucosidase during the extraction step before LC-MS/MS analyses) of the BSFL or their residual material after feeding on an AFB_1-contaminated diet might provide more insight. Furthermore, glutathione S-transferases (GSTs) are involved in one of the major AFB_1 detoxification pathways, namely the mercapturic acid pathway. Conjugation with mercapturic acid is a known detoxification mechanism in mammals and insects, and leads to the formation of a mercapturate which is generally more water soluble than its parent compound and is more readily excreted (Cooper and Hanigan 2018; Moss *et al.* 1985; Murcia and Diaz 2020a). Several insects use the mercapturic acid pathway as part of their adaptation strategy to the chemical defence mechanism of their host plant and insecticide resistance (Alias 2016; Sontowski *et al.* 2022). Furthermore, glycine N-methyltransferase (GNMT) was only upregulated after 6 h. GNMT possesses tumour suppressor effects against hepatocellular carcinomas in mice (Liao *et al.* 2009; Martínez-Chantar *et al.* 2008; Yen *et al.* 2009; Yen *et al.* 2013), and overexpression of GNMT seems to reduce AFB_1-DNA adduct formation and inhibits cancer cell proliferation (Yen *et al.* 2009). Overexpression of GNMT increased longevity in *D. melanogaster* (Obata and Miura 2015).

Phase III / Excretion

The main function of Phase III xenobiotic transporters is to excrete hydrophilic conjugates possibly formed during Phase I and II metabolism. Xenobiotic transport is done via membrane carriers which belong to two main clusters: ATP binding cassette (ABC) transporters, including the multidrug resistance protein (MRP) family, and solute carrier (SLC) transporters (Almazroo *et al.* 2017). ABC transporters have been found in various arthropod species, amongst which the mosquito *Anopheles gambiae* and the fruit fly *D. melanogaster*, and are associated with transport of and/or resistance to multiple acaricides and insecticides (Buss and Callaghan 2008; Dermauw and Van Leeuwen 2014). In the current study, upregulation of sodium-coupled monocarboxylate transporter 1 and multidrug resistance-associated protein lethal (2)03659 (*MRP2*) was also observed at 72 h. The sodium-coupled monocarboxylate transporter 1 is a transporter enzyme involved in the influx of xenobiotics into the cell (Phase 0 metabolism), and acts as a tumour suppressor gene in mammals (Hong *et al.* 2005; Li *et al.* 2003; Park *et al.* 2007).

MRP2 belongs to the ABC superfamily of transmembrane transport proteins. MRP2 protects cells from xenobiotics by extruding glutathione-, glucuronyl- and sulphate- conjugates from the

intracellular compartment (Lorico *et al.* 2002). Furthermore, MRP2 transports AFB_1-GSH conjugates of both the *endo*-isomers and *exo*-isomers in an ATP-dependent, osmotically sensitive manner, and is suggested to have a potential protective role in mammalian chemical carcinogenesis (Loe *et al.* 1997; Lorico *et al.* 2002). Though MRP2 is mainly known for its GSH transporting ability, it might play a role in the intestinal transcellular efflux of dietary glucosides (Walgren *et al.* 2000).

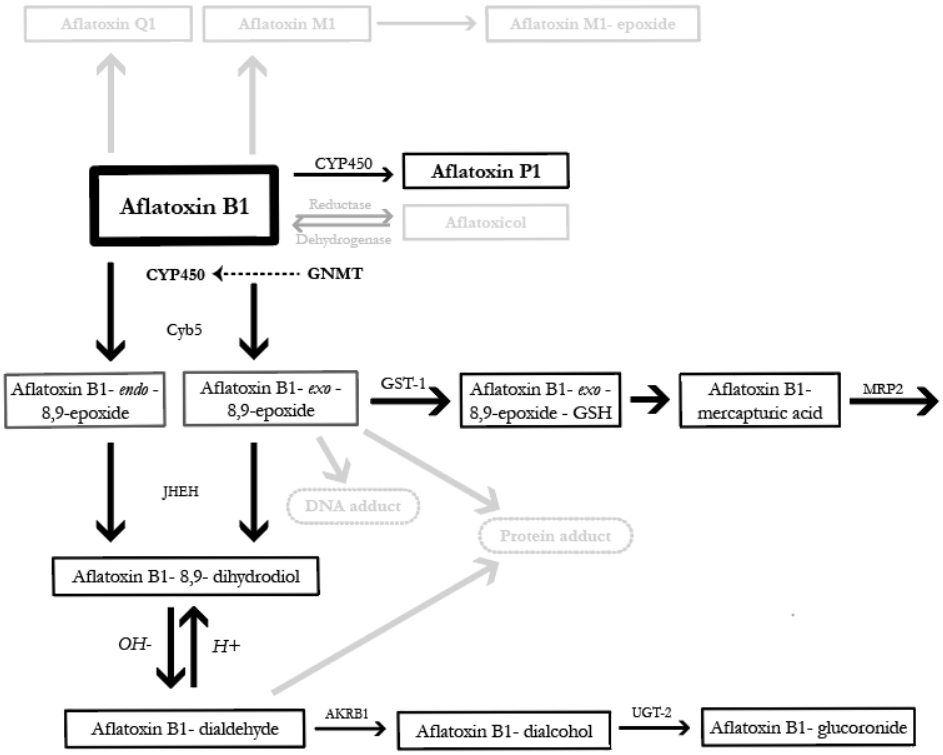

Figure 7. Potential metabolic pathway(s) in BSFL (in black) based on genes observed to be upregulated during AFB_1 feeding and their corresponding enzyme products. AKR1B1: aldo-keto reductase family 1 member B1; Cyb5: cytochrome b5; CYP450s: cytochrome P450 monooxygenase; GNMT: glycine N-methyltransferase; GSH: glutathione; GST-1: glutathione S-transferase 1; JHEH: juvenile hormone epoxide hydrolase 1; MRP2: multidrug resistance-associated protein lethal (2)03659; UGT-2: UDP-glucosyltransferase 2.

Growth and Development

Although we did not observe effects of AFB_1 exposure on survival or growth of the larvae, the transcription of multiple genes involved in larval growth was affected by exposure to AFB_1-contaminated diet. Genes encoding enzymes like trypsin, chymotrypsin and lipase that are

involved in proteolysis, lipid hydrolysis and other physiological processes were significantly downregulated (four-fold decrease in expression) in BSFL feeding on an AFB_1-spiked diet at 6 and 24 h. *In vitro* studies indicate similar negative effects of AFB_1 on enzymatic activity of trypsin and lipase in rats (Uwaifo 1980). Chymotrypsin-C (CTRC) is a peptidase known to degrade all human trypsin and trypsinogen isoforms and is involved in protein digestion. Carboxypeptidase B catalyses the hydrolysis of the amino acids arginine, lysine and ornithine. Genes coding for pupal cuticle protein, fatty acyl-CoA reductase CG5065, neuropeptide protein 31 that are involved in growth and developmental processes were also significantly downregulated across all timepoints in BSFL exposed to AFB_1-contaminated diet. Neuropeptides are important signalling molecules, whose absence or dysregulation can result in abnormal behaviour like uncoordinated movements and/or hyperactivity (Nelson *et al.* 1998). Dose-related decrease in neuropeptide production was observed upon AFB_1 feeding in rats (Trebak *et al.* 2015). Fatty acyl-CoA reductases (FARs) are involved in maintaining chemical communication in insects through production of short-range recognition pheromones as well as protecting the insect from desiccation (Finet *et al.* 2019).

Multiple studies have shown that BSFL do not exhibit any negative effects of AFB_1 on their survival and growth (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Meijer *et al.* 2019; Niermans *et al.* 2023b). Thus far, only one study has observed a slower development in BSFL upon provision of much higher (± 800 µg kg DM-1) concentration of AFB_1 as investigated in the current study (Heuel *et al.* 2023). Furthermore, exposure of other insect species to higher concentrations (ranging from 800 – 10,000 µg/kg) of AFB_1 resulted in negative effects on growth and development. Exposure of *Drosophila melanogaster* (strain Oregon R) to a high concentration (*i.e.* 10,000 µg/kg) of AFB_1 resulted in doubling of larval and pupal development time (Kirk *et al.* 1971). Furthermore, exposure to 800 µg/kg AFB_1 resulted in physical malformation (wings, leg and thorax) in 11% of adult *D. melanogaster* (Şişman 2006). Exposure to ≥ 2,000 µg/kg AFB_1 caused significant retardation in the development of larvae and pupae of *Spodoptera littoralis*, resulting in a significant reduction in the numbers of eggs laid and their hatching rate (Sadek 1996). Thus, oral uptake of very high AFB_1 concentrations by some insect species led to negative effects on insect growth and development; which was however not observed in BSFL.

Genes encoding proteins involved in the collagen biosynthesis pathway such as larval cuticle protein and endocuticle structural glycoprotein SgAbd-1 were significantly downregulated at all timepoints. In the nematode *Caenorhabditis elegans*, cuticular proteins are involved in their development and reproductive success and their downregulation negatively affects these processes (Yang *et al.* 2018). Although we did not observe any significant reduction in larval biomass in this study at the concentration applied, significant downregulation of growth and metabolic genes is indicative of negative long-term consequences in AFB_1-exposed larvae that

could potentially result in a shorter lifespan as adult or other developmental deformities and merits further investigations.

Immunity

Apart from genes associated with growth and development, genes known to contribute to insect immunity were also negatively affected. Interestingly, the dual oxidase (DUOX) gene was significantly downregulated at 6 h in AFB_1-fed BSFL. *DUOX* is responsible for production of reactive oxygen species (Huang *et al.* 2020) and maintaining gut homeostasis in insects (Jang *et al.* 2021). Downregulation of *DUOX* is indicative of negative gut homeostasis in BFSL resulting from exposure to AFB_1 diet. Phenoloxidase-2 was distinctly downregulated (LFC = -6.02) at 6 h in AFB_1 fed larvae. Phenoloxidases are key components of the insect immune system (González-Santoyo and Córdoba-Aguilar 2012). Similar down-regulation of phenoloxidase activity was also recorded in the mosquito *Aedes aegypti* when infected with the fungal pathogen *Isaria* sp. that produces mycotoxins (Ramirez *et al.* 2018). Fungal secondary metabolites like kojic acid (at 10^{-3} M) resulted in 80% inhibition of phenoloxidase activity in the cuticle and hemolymph of *Spodoptera frugiperda* (Dowd 1999). Genes coding for antimicrobial peptides like cecropin, glycine-rich DOT1, ctenidin-1 were significantly downregulated at all timepoints as a result of AFB_1 feeding. Downregulation of effector molecules may result in downregulation of the *imd* immune pathway in BSFL fed on AFB_1-spiked diet. In *D. melanogaster*, similar downregulation in expression of effector molecules was observed upon injection of destruxin A, produced by the entomopathogenic fungus *Metarhizium anisopliae* (Pal *et al.* 2007). The *imd* pathway is generally implicated to be responsible for immune defence again Gram-negative bacteria (Vogel *et al.* 2022). Downregulation of the components involved in the *imd* pathway would therefore imply that susceptibility against Gram-negative bacteria is enhanced. This suggests a trade-off for the humoral immune response of BSFL due to investment in detoxification pathways.

Conclusion

BSFL possesses a diverse set of genes that are upregulated upon exposure to AFB_1-spiked diet, allowing it to effectively metabolize and process AFB_1. Survival and larval biomass of BSFL were not significantly different from control treatments over a seven-day feeding trial. Based on the transcriptomic analysis, it is evident that a variety of detoxification/metabolic processes are upregulated in BSFL when fed on an AFB_1-spiked diet. Data from the current study indicate that AFB_1 might be transformed in BSFL via three of the major known AFB_1 detoxification pathways *i.e.* demethylation into AFP_1, glucosidation and conjugation with GSH, after which further transformation may occur via the mercapturic acid pathway.

At the same time, genes associated with growth, developmental and immunity processes are significantly downregulated. Although no larval mortality occurred in BSFL during the seven-day feeding trial at the concentration tested, potential trade-offs due to increased investments in

detoxification processes could result in higher susceptibility to infection by pathogens, low adult emergence, failure to emerge as adults or emergence of dysfunctional/malformed adults. Some genes (*cecropin, glycine-rich protein DOT1, ctenidin, acanthoscurrin*) that are involved in BSFL immunity pathways were downregulated. Impaired immunity could result in a lower resistance/increased susceptibility upon co-exposure to *e.g*. substrate-borne pathogens, which might affect BSFL survival and could consequently be detrimental in an insect rearing system.

Further, the genes encoding for multiple enzymes that are observed to be highly expressed on an AFB_1- contaminated diet (as hypothesized in Figure 7), merit functional validation using RNAi or CRISPR/Cas9 to confirm their role in AFB_1 metabolism/detoxification. Furthermore, the use of optimized extraction- and analytical methods to confirm the formation of the hypothesized metabolites may explain the missing fraction not obtained back in the mass balance analyses.

Acknowledgements

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Supplementary materials

Table S1
Sample quality and read-information

Treatment	Time (h)	Sample #	Trimmed read inputs	Mapped reads	Mapped reads %	Average input read length	Average mapped length
Control	6	1	19.5	16.8	86.13%	300	297.67
Control	6	2	10.0	9.3	93.02%	301	298.75
Control	6	3	21.4	19.7	92.22%	301	298.6
Control	6	4	14.3	13.4	94.02%	301	299.09
Control	24	5	21.0	19.4	92.63%	300	297.61
Control	24	6	21.3	18.1	85.24%	300	297.86
Control	24	7	20.2	18.7	92.76%	301	298.28
Control	24	8	12.9	11.5	89.35%	301	298.91
Control	72	9	12.2	10.6	87.31%	301	298.23
Control	72	10	22.8	21.1	92.30%	300	297.83
Control	72	11	21.6	20.3	93.78%	301	298.56
Control	72	12	11.2	9.7	86.66%	301	298.47
AFB_1	6	13	35.8	33.5	93.65%	289	286.63
AFB_1	6	14	25.2	23.5	93.00%	291	288.88
AFB_1	6	15	10.7	8.7	81.58%	301	298.77
AFB_1	6	16	12.4	11.6	93.90%	301	299.22
AFB_1	24	17	12.8	11.7	91.82%	300	298.07
AFB_1	24	18	15.2	14.5	95.19%	301	299.45
AFB_1	24	19	13.3	12.9	96.70%	301	299.58
AFB_1	24	20	16.7	16.0	96.29%	301	299.04
AFB_1	72	21	19.7	18.7	94.64%	301	298.84
AFB_1	72	22	17.6	16.1	91.56%	300	297.45
AFB_1	72	23	12.1	10.4	85.93%	301	297.38
AFB_1	72	24	12.2	10.8	88.65%	301	297.96
Average			17.2	15.7	91.18%		

Table S2
Instrumental MS/MS parameters of mycotoxins analysed in positive ionization mode

Component	Rt	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Aflatoxin B_1 (ql)	4.70	313.1	128.1	40	10	91	10
Aflatoxin B_1 (qn)	4.70	313.1	285.2	40	10	33	16
Aflatoxin B_1 (ql2)	4.70	313.1	241.0	40	10	54	15
Aflatoxin B_2 (ql)	4.40	315.1	259.2	40	10	43	18
Aflatoxin B_2 (qn)	4.40	315.1	287.2	40	10	37	18
Aflatoxin G_1 (ql)	4.1	329.0	200.0	40	10	53	12
Aflatoxin G_1 (qn)	4.1	329.0	243.2	40	10	39	14
Aflatoxin G_2 (ql)	3.8	331.1	245.2	40	10	43	14
Aflatoxin G_2 (qn)	3.8	331.1	313.2	40	10	35	18
Aflatoxicol (qn)	5.40	297.1	269.0	40	10	29	16
Aflatoxicol (ql)	5.40	297.1	114.9	40	10	81	12
Aflatoxicol (ql2)	5.40	297.1	141.0	40	10	65	14
Aflatoxin P_1 (qn)	4.20	299.0	271.0	40	10	33	18
Aflatoxin P_1 (ql1)	4.20	299.0	114.9	40	10	71	12
Aflatoxin P_1 (ql2)	4.20	299.0	90.9	40	10	67	10
Aflatoxin M_1 (qn)	3.84	328.9	272.9	40	10	33	18
Aflatoxin M_1 (ql)	3.84	328.9	229.0	40	10	55	16
Aflatoxin Q_1 (qn)	3.70	329.1	310.8	40	10	29	16
Aflatoxin Q_1 (ql)	3.70	329.1	177.0	40	10	45	22
Aflatoxin Q_1 (ql2)	3.70	329.1	128.0	40	10	67	14

qn: quantifier ions. ql: qualifier ions

Table S3
Recovery of the mycotoxins in the different matrices (substrate, residual material) analysed

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
Substrate	94%	100%	99%	106%	104%	94%	103%	103%
Residual material	81%	81%	88%	86%	90%	89%	73%	76%

Table S4
Limit of detection (LOD) and limit of quantification (LOQ) (µg/kg) per mycotoxin in the different matrices

Compound		AFB_1	AFB_2	AFG_1	AFG_2	AFP_1	AFQ_1	AFL	AFM_1
Substrate	LOD	< 0.24	0.13	0.08	0.07	0.28	0.26	0.38	0.38
	LOQ	< 0.24	0.26	0.12	0.37	0.94	0.85	1.27	0.85
Residue	LOD	< 0.24	0.16	0.09	0.09	0.3	0.35	0.44	0.54
	LOQ	< 0.24	0.33	0.14	0.46	0.99	1.16	1.47	1.21

Table S5
Overview of aflatoxin concentration (µg/kg) in the experimental diets

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
Control 1	nd	nd	nd	nd	nd	nd	nd	nd
Control 2	nd	nd	nd	nd	nd	nd	nd	nd
Control 3	nd	nd	nd	nd	nd	nd	nd	nd
Control 4	nd	nd	nd	nd	nd	nd	nd	nd
AFB_1 1	19.47	nd	nd	nd	nd	nd	nd	nd
AFB_1 2	20.12	nd	nd	nd	nd	nd	nd	nd
AFB_1 3	19.58	nd	nd	nd	nd	nd	nd	nd
AFB_1 4	21.80	nd	nd	nd	nd	nd	nd	nd
AFB_1 5	19.26	nd	nd	nd	nd	nd	nd	nd
AFB_1 6	20.16	nd	nd	nd	nd	nd	nd	nd
AFB_1 7	18.73	nd	nd	nd	nd	nd	nd	nd
AFB_1 8	18.54	nd	nd	nd	nd	nd	nd	nd

nd: not detected

Table S6
Survival and biomass (n=4) after 7 days of exposure to a control or AFB_1-contaminated diet

Treatment	Survival BSFL (%)	Biomass BSFL (mg)
Control 1	94.00	141.41
Control 2	99.00	151.08
Control 3	93.00	138.52
Control 4	90.00	125.04
AFB_1 1	93.00	128.55
AFB_1 2	91.00	135.02
AFB_1 3	88.00	142.53
AFB_1 4	90.00	143.03

Table S7
Larval biomass of samples collected for RNASeq experiment (DOL is day-old-larvae). Calculated average individual larval biomass (mg)

Treatment	5DOL	5DOL + 6h	6DOL	8DOL	11DOL
Control 1	22.25	25.90	54.60	130.00	141.41
Control 2	22.65	20.75	37.15	92.80	151.08
Control 3	22.30	27.05	37.55	118.65	138.52
Control 4	22.46	23.65	44.20	116.30	125.04
AFB_1 1	22.18	42.70	70.65	131.25	128.55
AFB_1 2	22.34	27.50	47.65	101.45	135.02
AFB_1 3	22.40	27.20	59.20	96.70	142.53
AFB_1 4	22.72	17.65	70.25	112.80	143.02

Biomass of 5DOL + 6h, 6DOL and 8DOL are based on the biomass of n=2 larvae per replicate.

Table S8
List of genes affected (significantly up- or down-regulated) by AFB_1 feeding

Time	Gene	log_2FC	Gene name	Function	Citation
6 h	LOC119646754	5.09	cytochrome P450 6a14	Xenobiotic detoxification	Berenbaum *et al.* (2021); Esteves *et al.* (2021); Liu *et al.* (2019a); Niu *et al.* (2008); Wamba *et al.* (2021); Wu *et al.* (2023); Zeng *et al.* (2007)
	LOC119660404	4.25	glycine N-methyltransferase	$AFB(_1)$ binds to the S-adenosylmethionine binding domain of GNMT Controls DNA methylation and epigenetic modulation of carcinogenic pathways in mice. GNMT knockout increases hepatocarcinoma susceptibility in mice	Liu *et al.* (2011); Martínez-Chantar *et al.* (2008); Yen *et al.* (2009)
	LOC119661368	3.52	juvenile hormone epoxide hydrolase 1	Coexpression of mEH with CYP resulted in significant decreases in measurements of AFB_1 genotoxicity Mitigate carcinogenic effects of AFB_1 in *Saccharomyces cerevisiae* model.	Kelly *et al.* (2002)
	LOC119648746	3.48	glutathione S-transferase 1	Modulates mercapturic acid pathways through conjugation	Cooper and Hanigan (2018)
	LOC119649556	2.75	aldo-keto reductase family 1 member B1	Converts protein-binding dialdehyde form of AFB_1-dihydrodiol to the nonbinding	Abraham *et al.* (2022); Barski *et al.* (2008); He *et al.* (2017); Knight *et al.* (1999);

			dialcohol metabolite. Oxidation of DON into 3-oxo-DON Modulates lipid synthesis, mitochondrial function and cellular apoptosis.	Wang *et al.* (2009)
LOC119651599	2.28	inosine triphosphate pyrophosphatase	Prevent incorporation of noncanonical purine nucleotides into DNA and RNA	Menezes *et al.* (2012)
LOC119646904	-28.35	glycine-rich protein DOT1	Dual function: Wing development and antimicrobial peptide activity	Baumann *et al.* (2010); Lorenzini *et al.* (2003); Zhong *et al.* (2006)
LOC119658421	-27.60	histidine-rich glycoprotein	regulate numerous important biologic processes, such as immune complex/necrotic cell/pathogen clearance, cell adhesion, angiogenesis, coagulation, and fibrinolysis	Poon *et al.* (2011)
LOC119651612	-16.57	mantle protein		
LOC119657463	-11.55	pupal cuticle protein	Role in development and reproduction ZEA induced-toxicity blocks collagen processing and cuticle formation	Yang *et al.* (2018)
LOC119659626	-9.24	chymotrypsin-2	Serine protease involved in proteolysis	
LOC119654864	-8.17	lipase 3	Reduced lipase activity in broilers during aflatoxicosis	Osborne and Hamilton (1981)

				In this case, downregulation suggests trade-off with other processes.	
	LOC119647198	-7.35	protein spaetzle 4	After proteolytic cleavage, acts a ligand for Toll receptor that is involved in immunity responses Modulates innate immunity responses	Jang *et al.* (2006)
	LOC119658699	-6.03	phenoloxidase 2		Dowd (1999)
	LOC119652329	-2.66	dual oxidase	Mediates gut symbiosis and tracheal network stability in *Riptortus pedestris* Regulates intestinal bacterial homeostasis in *Hermetia illucens*	Huang *et al.* (2020); Jang *et al.* (2021)
24 h	LOC119658527	6.04	carboxypeptidase B	Hydrolysed OTA into non-toxic OTα and L-phenylalanine	Chang *et al.* (2015)
	LOC119646979	4.52	cytochrome b5	Processing of xenobiotic compounds	Berenbaum *et al.* (2021); Dunkov *et al.* (1997); Elzaki *et al.* (2019); Saner *et al.* (1996)
	LOC119646754	3.55	cytochrome P450 6a14		
	LOC119648928	3.19	cytochrome P450 6a13		
	LOC119649844	2.27	UDP-glucosyltransferase 2	Conserved across multiple kingdoms Phase II detoxification enzyme Improves resistance against DON and NIV Convert zearalenone into ZEN-4-O-glucoside Improves resistance against spirotetramat in *Aphis gossypii* Glover	Bock (2016); Khairullina *et al.* (2022); Pan *et al.* (2020); Poppenberger *et al.* (2006)

	LOC119646589	2.19	juvenile hormone epoxide hydrolase 1		
	LOC119649056	-26.71	cuticle protein 16.5		
	LOC119659626	-16.15	chymotrypsin-2		
	LOC119658421	-15.33	histidine-rich glycoprotein	Antifungal activity conferring innate immunity	Poon *et al.* (2011); Rydengård *et al.* (2008)
	LOC119646904	-14.53	glycine-rich protein DOT1	In *Sorghum*, generates innate immune response upon biotic stress Antimicrobial activity	Halder *et al.* (2019)
	LOC119657474	-11.62	ctenidin-1-like	Antimicrobial peptide from spider haemocytes	Baumann *et al.* (2010)
	LOC119657650	-5.58	cecropin-like peptide 1	Confers immunity against Gram negative bacteria	
72 h	LOC119647513	6.63	esterase E4-like	Increases insecticide resistance in *Myzus persicae* Increased pesticide resistance in *Sitobion avenae* Fabricius	Blackman *et al.* (1996); Field and Devonshire (1998); Li *et al.* (2007); Zhan *et al.* (2020)
	LOC119658527	6.35	carboxypeptidase B	Degradation and detoxification of OTA	Chang *et al.* (2015)
	LOC119657973	5.30	cytochrome P450 307a1		
	LOC119654998	4.94	cytochrome P450 307a1		
	LOC119649263	2.82	glutathione S-transferase 1		
	LOC119661114	2.78	cytochrome P450 CYP12A2		
	LOC119657136	2.29	cytochrome P450 CYP12A2		
	LOC119656501	2.17	cytochrome P450 4d14		
	LOC119657225	-16.61	endocuticle structural glycoprotein SgAbd-1	Responsible for endocuticle formation	Ye *et al.* (2021)

LOC119657545	-12.61	acanthoscurrin-1	Glycine-rich antimicrobial peptide from tarantula spider *Acanthoscurria gomesian* Constitutively expressed in haemocytes and released to plasma following an immune challenge with *E. coli* and *M. luteus*	Lorenzini *et al.* (2003)
LOC119649056	-11.09	cuticle protein 16.5	Cuticle formation and development	
LOC119657474	-10.85	ctenidin-1		
LOC119657589	-4.91	cecropin-like peptide 1		

Table S9
Genes distinctly upregulated at two distinct timepoints

Common timepoints	Gene ID	Gene Name	log_2FC	Function
6 / 24 h	LOC119646754	cytochrome P450 6a14	5.09 / 3.55	Detoxification of xenobiotic compounds (Berenbaum *et al.* 2021)
	LOC119653112	uncharacterized LOC119653112	4.79 / 4.29	
	LOC119653108	uncharacterized LOC119653108	4.57 / 3.52	
	LOC119653109	uncharacterized LOC119653109	3.57 / 3.81	
	LOC119660515	fatty acid-binding protein	2.88 / 2.66	Iwashita and Nagashima (2011)
	LOC119661115	PI-stichotoxin-She2a-like	2.75 / 3.04	Homologous to Kunitz-type serine protease inhibitors (Madio *et al.* 2019) Serine protease inhibits chymotrypsin, trypsin and pepsin enzyme (Park *et al.* 2005)
	LOC119657935	ring canal kelch homolog	2.34 / 2.30	Maintain cytoplasm transport and ring canal organization during oogenesis in *Drosophila* (Robinson and Cooley 1997)
	LOC119646589	juvenile hormone epoxide hydrolase 1	2.24 / 2.19	Catalyses the addition of water to epoxides and arene oxides to give vicinal diol products (Tzeng *et al.* 1996)
24 / 72 h	LOC119657336	extensin-3	7.36 / 6.73	
	LOC119658527	carboxypeptidase B	6.04 / 6.35	Degradation of ochratoxin A by carboxypeptidase A and engineered carboxypeptidase (Chang *et al.* 2015; Hu *et al.* 2018)
	LOC119661758	uncharacterized LOC119661758	4.17 / 4.66	
	LOC119659847	uncharacterized LOC119659847	2.73 / 3.85	
6 / 72 h	LOC119659392	uncharacterized LOC119659392	2.45 / 3.01	

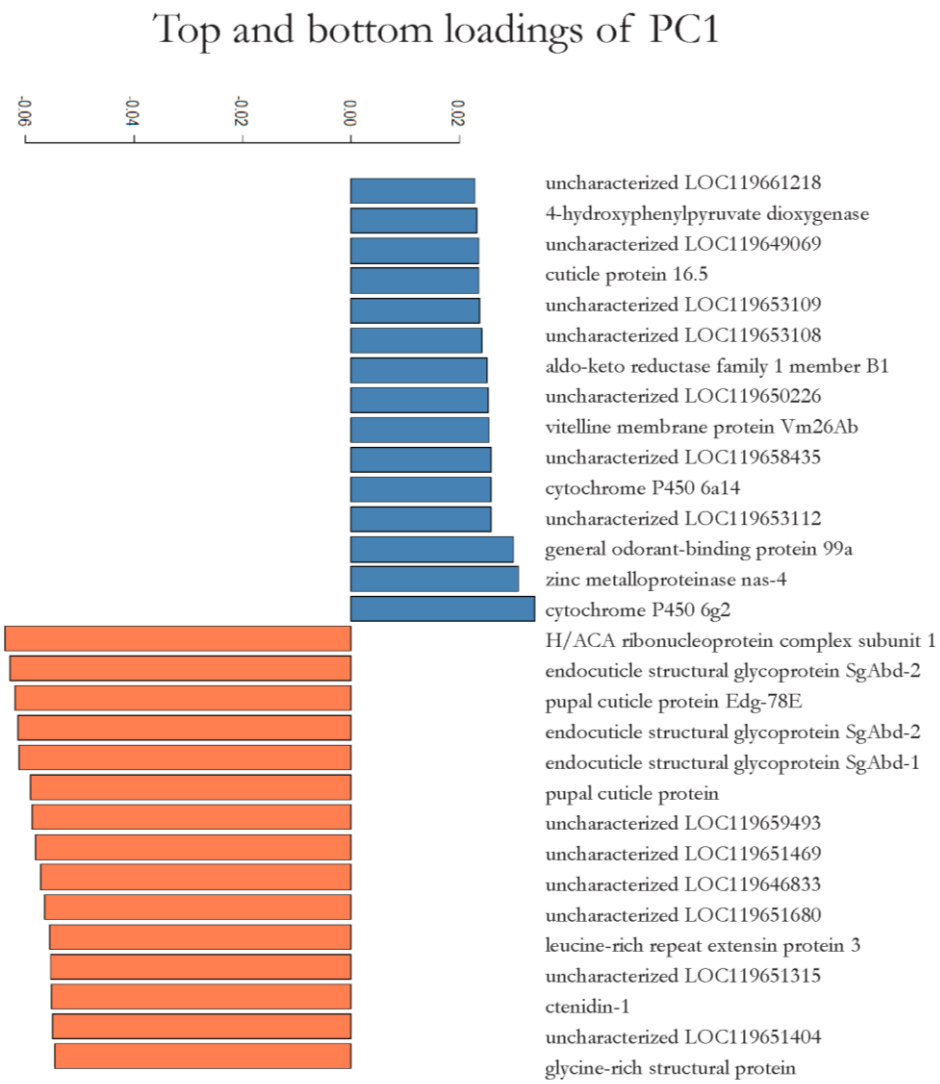

Figure S1
Genes contributing to top (*i.e.* positive axis, in blue) and bottom (*i.e*. negative axis, in orange) loadings of PC1 axis of PCA plot

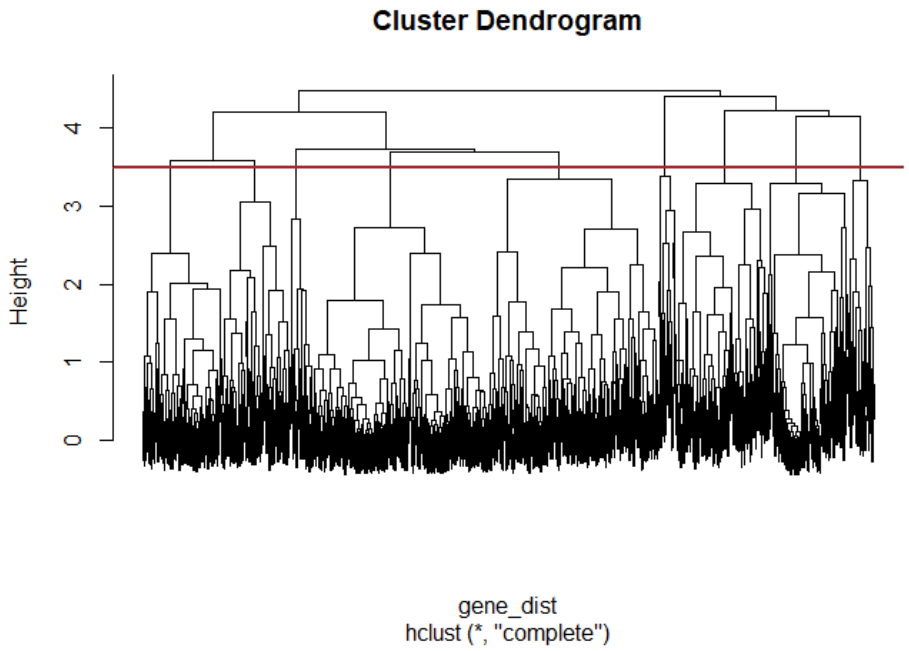

Figure S2
Hierarchical clustering of DEGs into nine clusters.

Chapter 7

Conversion of aflatoxin-contaminated groundnut (*Arachis hypogaea* L.) press cake by larvae of black soldier fly *Hermetia illucens* results in a complete mass balance for aflatoxin B_1

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Submitted

Abstract

Groundnuts (*Arachis hypogaea* L.) are considered as one of the most important cultivated food crops globally. They are especially valued for their protein and fat content and therefore play an important role in food security and traditional cuisines. Groundnuts are used for vegetative oil production, which generate a variety of by-products, such as peanut press cake (PPC) which consists of the solids remaining after pressing. Groundnuts are sensitive to infection with aflatoxigenic fungi during production. Aflatoxins are highly toxic to both humans and livestock, and contaminated crops containing aflatoxin concentrations above a legal maximum are deemed unsafe for consumption and trade. Innovative aflatoxin management strategies are needed and the insect production sector could possibly offer one of these solutions.

In the current study, larvae of the black soldier fly (BSFL; *Hermetia illucens* L.) were exposed to a PPC diet naturally contaminated with aflatoxins. After an exposure lasting eleven days, data on larval survival and biomass were collected. The PPC, BSFL and the residual material were analysed by LC-MS/MS to determine the concentration of eight different aflatoxins and known metabolites. Additionally, a bio-accumulation factor and a molar mass balance were calculated. BSFL survival and biomass were not affected by exposure to the aflatoxin-contaminated PPC diet. The aflatoxins did not accumulate in the insect body, which provides a promising outlook for the safety of rearing insects on aflatoxin contaminated PPC with the purpose of using them as food- and/or feed. Aflatoxin B_1 (AFB_1) was the dominant compound found in PPC. Formation of aflatoxicol, aflatoxin P_1, and aflatoxin M_1 occurred and taking these metabolites into account, a complete molar mass balance, thus full recovery, for AFB_1 was obtained. This result differs from previous studies using artificially spiked substrates in which 17-38% was recovered. According to our knowledge, this is the first study that documents complete recovery of AFB_1 after bioconversion. This finding calls for additional studies comparing naturally contaminated with artificially spiked feedstuff to establish if the assumption that artificial spiking is representative for natural contamination is valid.

Introduction

Groundnuts, also known as peanuts (*Arachis hypogaea* L.), belong to the most valued cultivated food crops worldwide. Groundnuts are especially valued as a source of protein, fat, and energy and play an important role in food security (Asare Bediako *et al.* 2019), the elimination of malnutrition (Guimón and Guimón 2012) and are a common ingredient in traditional cuisines (Kaaya *et al.* 2006; Nugraha *et al.* 2018). Additionally, groundnuts are an important income-generating commodity for many smallholder farmers in especially sub-Saharan Africa and Asia (Diop *et al.* 2004). Commercially, groundnuts are used for oil production, but apart from oil, groundnuts are also used as confections for the production of peanut butter and as ingredient in drinks, soups, and desserts (Arya *et al.* 2016). A major by-product during oil production is

peanut press cake (PPC), which is a product composed of the solids remaining after groundnuts have been pressed to collect the oil (Zhao *et al.* 2015).

Groundnut cultivation faces numerous challenges such as drought, infection of the plants by diseases and pest infestations. Plant stress induced by drought, high soil temperatures, and damage caused by insect feeding might lead to the invasion of aflatoxigenic fungi (Torres *et al.* 2014). Aflatoxin contamination following fungal infection is influenced by a variety of intrinsic, extrinsic and processing factors at both the pre- and post-harvest stage (Asare Bediako *et al.* 2019). Aflatoxin contamination is found in many regions around the world, but is especially prominent in many tropical and subtropical regions as climate conditions in these regions (temperature and humidity) are optimal for fungal growth and aflatoxin production (Adetunji *et al.* 2021; Kamika *et al.* 2014).

Aflatoxins are highly toxic and cause disease in humans and livestock (Lien *et al.* 2019). The International Agency for Research on Cancer (IARC) concluded that aflatoxin B_1 (AFB_1) should be considered as a Group 1 carcinogen producing liver cancer in humans (IARC 1993). Acute aflatoxicosis, caused by the consumption of moderate to high levels of aflatoxins, can cause haemorrhage, acute liver damage, and death (Bbosa *et al.* 2013b). Outbreaks of human illness caused by aflatoxins have been occurring worldwide, and the outbreak of aflatoxin poisoning in the Eastern and Central province of Kenya during January-July 2004 is just one example (CDC 2004; Lewis *et al.* 2005). More commonly, chronic subacute exposure to aflatoxins occurs (Kaaya *et al.* 2006). Chronic aflatoxicosis has been reported to cause an impaired immune function (Zain 2011), stunted growth in children, and the development of hepatocellular carcinomas, amongst others (Bbosa *et al.* 2013b; Meijer *et al.* 2021b). Besides the effects aflatoxin exposure has on humans, aflatoxin contamination has been linked to increased mortality of farm animals as well (Cao *et al.* 2022; Zain 2011).

The above mentioned health effects resulting from aflatoxin exposure make aflatoxin-contaminated crops less valuable and considered as unsafe for consumption and trade and as a consequence cannot enter the food chain. Consequently, many countries have set maximum levels (ML) of total aflatoxin in groundnut- or groundnut products intended for human and animal consumption, preventing entrance to international markets (Asare Bediako *et al.* 2019). Worldwide, the MLs set for aflatoxins for human consumption range from 4 to 30 µg/kg (Udomkun *et al.* 2017). The European Union (EU) has set the ML for the presence of aflatoxins (sum of AFB_1, B_2, G_1 and G_2) in groundnuts directly suitable for human consumption at 4 µg/kg (EC 2006c) and the ML for AFB_1 in all feed materials at 20 µg/kg (EC 2002a).

Aflatoxin concentrations in groundnut (products), in reality often exceeds the set MLs (Adetunji *et al.* 2021). Lien *et al*. (2019) collected 1089 samples of groundnut and groundnut products (candy, butter, etc.), originating from a variety of countries, of which 25% tested positive for

aflatoxins (Lien *et al.* 2019). Different levels of AFB_1, AFB_2, AFG_1 and AFG_2 were also detected in groundnut (product) samples in a variety of African counties. Aflatoxins were found in almost 50% of the samples from Southern African counties, while the majority (almost 100%) of the groundnut samples from West African States were contaminated (Adetunji *et al.*). Total aflatoxin concentrations in groundnut originating from Botswana and Zimbabwe ranged from 12 to 329 µg/kg (Mphande *et al.* 2004) and 9 to 698 µg/kg (Maringe *et al.* 2017), respectively. Total aflatoxin concentrations in raw groundnuts originating from the Democratic Republic of Congo varied from 2 to 1258 µg/kg (Kamika *et al.* 2014). The AFB_1 concentration in peanut (products) collected in Indonesia varied from 0 (below limit of detection) to 357 µg/kg (Nugraha *et al.* 2018).

As a result innovative aflatoxin management strategies are needed (Udomkun *et al.* 2017) and the insect production sector could possibly offer such a strategy (*Chapter 2*; Niermans *et al.* 2021). Multiple studies have shown that vital biological variables, *i.e.* growth and survival, of black soldier fly larvae (BSFL; *Hermetia illucens* L.) fed on an aflatoxin-contaminated diet are not affected at all (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Meijer *et al.* 2019; Niermans et al. 2023b). These studies also showed that aflatoxin breakdown and metabolism in insects occur, and that AFB_1 and its related metabolites were mostly absent from the insects themselves. Studies like these were mostly performed with aflatoxin-spiked materials in a laboratory setting, but it is essential to study what happens when the BSFL are exposed to naturally contaminated materials. This study aimed to determine the fate of aflatoxins in BSFL fed on a diet of PPC that was naturally contaminated with aflatoxins. Secondary aims were to investigate if aflatoxin-contaminated PPC could serve as a suitable substrate for rearing BSFL as a safe source of insect protein.

Materials and methods

Experimental set-up

In this experiment, BSFL (originating from the colony of InsectoCycle, Wageningen, the Netherlands) were fed on either a control PPC diet or on a naturally aflatoxin-contaminated PPC diet. The final BSFL feed consisted of either clean- or aflatoxin-contaminated PPC (35%) and water (65%).

One-hundred individual five-day-old BSFL (average individual starting live weight 2,8 mg) were placed on 265 g of the prepared feed in a ventilated rearing container (2x3 raster size, 14.9 x 9.7 x 5.9 cm, Auer packaging, Schiphol-rijk, the Netherlands), and placed in a climate chamber at 28 ± 1°C, 60% relative humidity and a day/night rhythm of 12/12 h. Exposure was performed in triplicate for the control PPC treatment and with six replicates for the contaminated PPC treatment. The water content of the PPC diet was kept at 65% by adding water on a regular basis during the experiment. Exposure ended after eleven days and BSFL were separated from the residual material (left over substrate and frass) manually. No gut cleaning step *e.g*. transfer to

clean feed was implemented after harvesting, and BSFL were neither washed nor dried after harvesting. Sample material of both the larvae and the residual material was collected, placed in plastic bags, and frozen at -18°C until analyses.

Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1); Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

Extraction procedure

In order to obtain a fine powder, frozen BSFL were grinded under liquid nitrogen. The powder was stored at -80°C until analyses. No pre-treatment was performed on the substrate- and residual material. Extractions were performed as described by Camenzuli *et al*. (2018) and were based on an in-house method from Wageningen Food Safety Research which is validated according to SANTE/12682/2019 (EC 2019b). Extractions were done with 1 g of larval sample and the extraction procedure was adjusted accordingly. After extraction, each extract was prepared with- and without the addition of a standard mix containing AFB_1, AFB_2, AFG_1, AFG_2, AFL, AFP_1, AFM_1 and AFQ_1. The preparation without standard addition was as follows; 200 µL sample extract was mixed with 200 µL water in a syringeless PTFE filter vial (Mini-UniPrep, Whatman, Marlborough, MA). For the extracts with standard addition, 200 µL of sample extract was added together with 190 µL water and 10 µL of the standard mix in a second syringeless PTFE filter vial (Mini-UniPrep, Whatman, Marlborough, MA). The files were capped, vortexed and placed in the refrigerator for 30 min. After 30 min the vials were closed and stored at 4°C until LC-MS/MS analyses.

LC-MS/MS analyses

The LC-MS/MS system, the process of LC-MS/MS analyses, LC separation, eluents used and the conditions set for electrospray ionization were performed as described in *Chapter 4* (Niermans *et al.* 2023b) except that, in the current study, analyses were solely performed in positive ESI mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Table S1).

Data analyses

The data on growth and biomass of the BSFL, collected after eleven days of exposure, was analysed with a Mann-Whitney test with a significance level (α) of 0.05 (GraphPad Prism v4). LC-MS/MS data was analysed with SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA). Peaks were identified as based on criteria for identification and recovery mentioned in SANTE/12682/2019 (EC 2019b). Analysed concentrations were corrected for recovery

percentages (EC 2006a) of their respective aflatoxin or known metabolites. The recovery percentages can be found in Table S2. Concentrations were calculated by dividing the peak area of the sample by the area of the sample with standard addition minus the peak area of the sample, and multiplied by the addition level of the respective mycotoxin. A limit of detection (LOD) and limit of quantification (LOQ) of each aflatoxin and metabolite were determined based on a calibration in solvent and adjusted for the recovery (%) of the respective matrices (Table S3). The LOD was determined as the lowest concentration that complied with the following criteria: retention time (r_t) may not deviate more than ± 0.1 min relative to the retention time as measured in the standard solution, ion ratio should be within ± 30% of the average ion ratio of the standard solution and the signal to noise ratio (S/N) should be ≥ 3. The LOQ was determined following the same criteria but the S/N had to be ≥ 10. For further analyses, mycotoxin (metabolite) concentrations below the LOQ were set at 0 as these values are not considered suitable for use in calculations. Replicate 2 was analysed in triplicate as during the first analysis the AFB_1 concentration was outside of the quantification range. The average concentration of the second and third analysis was used as the concentration of replicate 2. Sample homogeneity was determined by calculating the relative standard deviation for the aflatoxin concentration in the PPC diets (n=6). A molar mass balance was calculated as described in *Chapter 3* (Niermans *et al.* 2023a), and was done for the average concentrations determined over the replicates (n=6) The molecular weight of the aflatoxins used for these calculations are shown in Table S4. Figures were made in GraphPad Prism v4.

Results

Larval survival and biomass

The average survival of the BSFL varied from 90 to 93% in the PPC control diets and between 89 and 97% in the contaminated PPC diet. Although variation was higher as compared to the control diet, feeding BSFL an aflatoxin contaminated PPC diet did not significantly affect survival. The average individual larval biomass varied between 0.20 and 0.23 g for the control diet and between 0.20 and 0.24 g for the contaminated diets and did not differ significantly between the two diets.

Aflatoxin bioaccumulation and metabolism

The PPC control samples contained low aflatoxin concentrations of around 1.02 ± 0.66 µg/kg AFB_1 and 0.28 ± 0.13 µg/kg AFG_1. The contaminated PPC diets contained more than 10 times higher concentrations of AFB_1 (14.66 ± 5.28 µg/kg) and four times higher concentrations of AFG_1 (1.14 ± 0.50 µg/kg) than the control diets. Additionally AFB_2, AFG_2, and AFM_1 were detected in the contaminated PPC diet (Table 1). The contaminated PPC samples exhibited considerable variation in concentration indicating heterogeneity in aflatoxin distribution in the substrate. The

relative standard deviation of the aflatoxin (AFB_1, AFB_2, and AFG_1) concentration in the PPC varied between 36% and 61%.

Table 1. Average and standard deviation (SD) of the aflatoxin concentrations (in µg/kg) in the PPC diet, BSFL and residual samples after eleven days of feeding on either the control or aflatoxin-contaminated (cont.) diet

	AFB_1		AFB_2		AFG_1		AFG_2	
Sample Name	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
PPC control	1.02	0.66	0.11	0.19	0.28	0.13	< LOQ	-
PPC aflatoxin-cont.	14.66	5.28	3.44	2.09	1.14	0.50	0.12 [1]	-
BSFL control	0.85	1.26	< LOQ	-	< LOQ	-	< LOQ	-
BSFL aflatoxin-cont.	0.46	0.31	< LOQ	-	< LOQ	-	< LOQ	-
Residue control	5.79	5.94	0.73	0.25	0.45	0.08	< LOQ	-
Residue aflatoxin-cont.	14.61	4.64	2.77	0.85	1.09	0.62	< LOQ	-

	AFL		AFP_1		AFM_1		AFQ_1	
Sample Name	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
PPC control	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
PPC aflatoxin-cont.	< LOQ	-	< LOQ	-	0.27 [1]	-	< LOQ	-
BSFL control	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
BSFL aflatoxin-cont.	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
Residue control	< LOQ	-	2.83	1.09	< LOQ	-	< LOQ	-
Residue aflatoxin-cont.	0.69	0.77	2.43	0.55	0.69	0.55	< LOQ	-

< LOQ: concentrations in all replicates of the control (n=3) or contaminated (n=6) diet were below the limit of quantification. [1] Detected in only one of the replicates. Residue: residual material. An overview of the aflatoxin concentrations per replicate can be found in Table S5.

In BSFL fed contaminated PPC a concentration of AFB_1 0.46 ± 0.31 µg/kg was found. None of the other aflatoxins or metabolites were detected in quantifiable concentrations in the BSFL. The levels of AFB_1 found in the larvae are lower than the levels found in the substrate, demonstrating that no bioaccumulation occurred.

The average calculated molar mass balance for the total aflatoxins was 99 ± 26%, indicating a full recovery of the initially present amounts of aflatoxins (Figure 1).

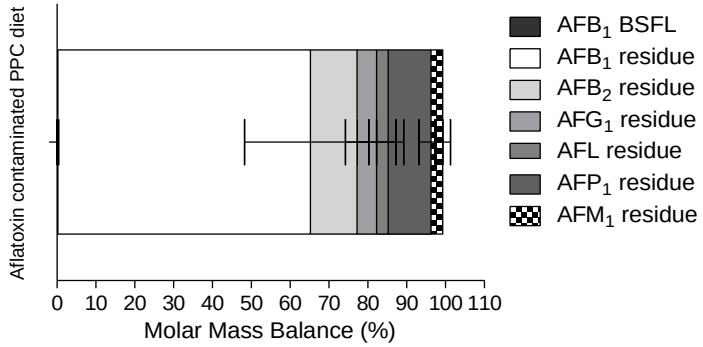

*Figure 1***.** Molar mass balance of the aflatoxins for BSFL fed on a aflatoxin-contaminated diet. Error bars represent the SD as a measure of variability between the replicates (n=6). Residue: residual material. An overview of the average contribution of the aflatoxins to the overall molar mass balance can be found in Table S6.

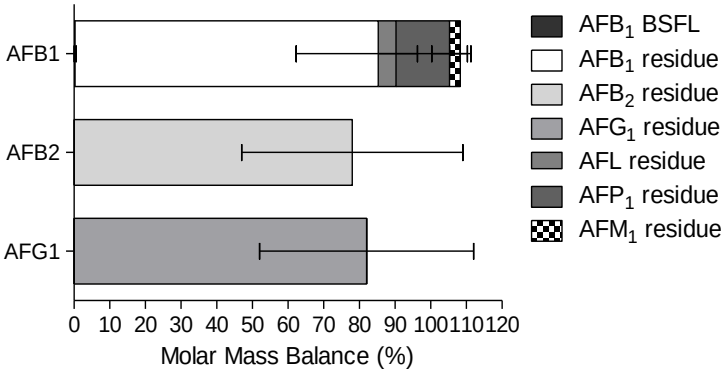

*Figure 2***.** Molar mass balance of AFB1 and its related metabolites, AFB2 and AFG1 for BSFL fed on a aflatoxin contaminated diet. Error bars represent the SD as a measure of variability between the replicates (n=6). Residue: residual material.

Based on their chemical structure, aflatoxins are considered difuranocoumarin derivatives, and can be divided into two chemical series: 1) difurocoumarocyclopentenone: AFB_1, AFB_2, AFM_1, AFL; and 2) difurocoumarolactone: AFG_1 and AFG_2 (Bbosa *et al.* 2013a). Aflatoxins from the B-group are generally not precursors of the G-group aflatoxins, however, aflatoxins from both groups can be independently produced by fungi in the same substrate (Yabe *et al.* 1999). Additionally, AFB_2 is in general not considered as a metabolic product of AFB_1 (Bbosa *et al.* 2013a); we therefore considered it relevant to separate the contribution of the aflatoxins. This separation resulted in a molar mass balance of 108 ± 29% for AFB_1 and its related metabolites,

and 78 ± 31% for AFB_2, while this was 82 ± 30% for AFG_1 (Figure 2).AFB_1 metabolism seems to occur after ingestion of PPC by BSFL as the initial presence of AFB_1, and AFM_1 in the contaminated PPC resulted in the presence of AFB_1, AFL, AFP_1 and AFM_1 in the contaminated PPC residual material. AFQ_1 was not found in any of the materials. Taking the metabolites into account, a full recovery of AFB_1 was found.

Discussion

In the current study AFB_1 was detected in a low concentration in BSFL, but AFB_1 was either not detected in concentrations above the LOD (Bosch *et al.* 2017), LOQ (Camenzuli *et al.* 2018) or had a bioaccumulation factor of zero (*Chapter 4*; Niermans *et al.* 2023b) in previously conducted studies (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Niermans *et al.* 2023b). The difference between our findings and previous study results can be explained by the fact that in the current study, BSFL were not presented clean feed after feeding on the PPC diets, to allow gut cleaning, and were not washed after harvesting. Possibly, the small amount of AFB_1 detected in the larval material resulted from the presence of contaminated material in their gut, or on their cuticle. The concentration of AFB_1 detected in the larval materials was, however, far below the ML of AFB_1 (20 µg/kg) in all feed materials for animal feed mentioned in the EU Directive 2002/32/EC (EC 2002a).

In this study, the aflatoxins AFB_2, AFG_1, AFG_2 (one replicate), AFL, AFP_1 and AFM_1 were detected in the residual material. AFP_1, AFQ_1 and AFL are not mentioned in any of the food and feed related regulations. Therefore, they are often exempt from (routine) analyses and not included in the analyses reported in the literature. In some of the residual material of BSFL fed an AFB_1 contaminated diet a signal (above the LOQ) for AFP_1 was detected, but this signal could not be expressed in concentrations due to matrix interferences (Camenzuli *et al.* 2018). Also, AFL was detected in the residual material of substrates spiked with high concentrations of AFB_1 (Camenzuli *et al.* 2018). Furthermore, a recently conducted study also showed the formation of AFP_1 after feeding BSFL on an AFB_1 spiked diet (*Chapter 4*; Niermans *et al.* 2023b). AFM_1 was also detected in the residual material in the current study, which is interesting as AFM_1 is a hydroxylated metabolite of AFB_1 and is mainly found in milk or milk products obtained from livestock that have ingested contaminated feed (Akinyemi *et al.* 2022; Tozzi *et al.* 2016). Other studies also detected AFM_1 in groundnut (product) samples (Campbell *et al.* 2017; Huang *et al.* 2010), and very low concentrations of AFM_1 were also detected in the residual material of *Alphitobius diaperinus* Panzer larvae after being fed an AFB_1 contaminated diet (Camenzuli *et al.* 2018). Overall, the formation of the AFB_1 metabolite AFP_1 has been observed in BSFL feeding studies with spiked (Camenzuli *et al.* 2018; *Chapter 4*; Niermans *et al.* 2023b) as well as naturally contaminated material (current study). It therefore seems that AFP_1 is a BSFL-specific metabolite either formed by larval CYP enzymes or larval microbiota. On the other hand, this is the first time that AFM_1 is found in a study where BSFL were fed an AFB_1 contaminated diet. AFM_1 was

previously detected in other groundnut (product) samples, which might indicate that its formation is initiated by the microorganisms present in the groundnut substrate.

The molar mass balance calculated in the current study indicated a complete recovery (99 ± 26%) of the initially present aflatoxins, however, variation between the replicates was high. This finding is in contrast with previous reports in which AFB_1 mass balances indicated an unrecovered fraction of > 83% (Bosch *et al.* 2017; Camenzuli *et al.* 2018). The calculated molar mass balance in another study with spiked AFB_1 indicated a missing fraction of 38% (*Chapter 4*; Niermans *et al.* 2023b), which is higher, but more in line with the current study when taking the observed variation into account. Data from the current study indicates a difference between aflatoxin mass balances when BSFL were fed a spiked or a naturally contaminated diet. It therefore seems relevant to investigate whether this difference is systematic. Additionally, the current study showed that, when calculating a molar mass balance for the aflatoxins AFB_1, AFB_2 and AFG_1 separately, a small (< 22%) missing fraction, and a big variation between the replicate samples was found for AFB_2 and AFG_1, while the mass balance of AFB_1 was complete. Previous studies (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Niermans *et al.* 2023b) only performed (molar) mass balance calculations for diets spiked with AFB_1. So far, no spiking studies with other aflatoxins (AFB_2, AFG_1, AFG_2) were performed. Based on the results of the current study, it seems that (almost) no metabolism of these aflatoxins takes place. In view of the small number of studies performed thus far, additional research is warranted.

To our knowledge, no studies in which BSFL were fed a diet naturally contaminated with aflatoxins has been performed before, but such studies that calculated mass balances are available for either other insect species or other mycotoxins. In one study, BSFL were fed a diet which was naturally contaminated with other mycotoxins (Leni *et al.* 2019). The latter authors fed BSFL on a diet of corn distillate residues naturally contaminated with deoxynivalenol (DON), fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), and calculated a mass balance of 81% for DON and 72% for FB_1. When comparing this to the spiked situation, a much lower mass balance (39%) was calculated when BSFL were fed on a diet in which DON was spiked at a level of 1x the guidance value (GV) set for animal feed (Camenzuli *et al.* 2018; EC 2006b). However, when DON was spiked at the same concentration but the diet also contained spiked concentrations of AFB_1, ochratoxin A (OTA) and zearalenone (ZEN) a mass balance of 80% was calculated (Camenzuli *et al.* 2018). Another study calculated a molar mass balance for FB_1, spiked at a level of 0.1x the GV set for animal feed (EC 2006b), of 72%, which is the same as calculated in the above mentioned study (*Chapter 4*; Niermans *et al.* 2023b). Based on the previously mentioned data it seems that mycotoxin recovery based on mass balance calculation is affected by the specific mycotoxin and by the type of contamination, *i.e.*, naturally contaminated or spiked. Furthermore, these results indicate a possible effect of the type of diet on the mass balance and the possibility for insects to metabolise or excrete mycotoxins. Different feed substrates also contain different

microorganisms (bacteria, fungi, yeasts) and the role of these substrate-specific microorganisms in, for example, AFB_1 degradation/metabolism needs to be further studied.

It is known that cytochrome P450 monooxygenase enzymes play an important role in AFB_1 metabolism (Bbosa *et al.* 2013a), and their role in mycotoxin metabolism in a variety of insects has also been demonstrated (Berenbaum *et al.* 2021). Interactions between enzymes, *e.g.* (competitive) inhibition, could occur (Korzekwa 2014) resulting in competition for metabolism when multiple mycotoxins are present in the substrate (naturally contaminated and spiked) which may saturate the enzymes needed for metabolism. Furthermore, it is possible that the mycotoxins secreted by living fungi in the plant tissue are differently bound to the plant matrix, and protection of the mycotoxin by this matrix would be different when spiked (Schaarschmidt and Fauhl-Hassek 2021). Additionally, when spiked, a larger fraction of the mycotoxin may remain in 'free' form (not bound to *e.g.* the plant matrix) and is thereby more accessible for enzymatic breakdown. It is therefore advised to perform an experiment in which BSFL are fed on spiked as well as naturally contaminated PPC material to test whether the outcome of this study is representative. Since no completely aflatoxin-free PPC material was available, a comparison between spiking and natural contamination was not an option in the current study.

When performing a feeding study, photochemical or microbial degradation of the tested compounds could take place over time (Albert and Muñoz 2022). A spiked diet is usually prepared right before the start of the experiment, whereas the PPC diet used in the current study was stored for a longer period of time. Photochemical or microbial degradation of the aflatoxins in the PPC diet could have, for example, already taken place, leading to a stable aflatoxin concentration before the start of the feeding study. Furthermore, it needs to be mentioned that when working with naturally contaminated materials, it might be possible that fungal spores of aflatoxigenic species might still be present. This could result in additional formation of mycotoxins during the experiment, which might have led to the formation of the complete molar mass balance. However, when appropriately pre-treated, it is assumed the spores are inactive (Kutasi *et al.* 2021). As the PPC used in the current experiment was pre-treated, it seems unlikely active fungal spores were still present in the current experiment.

Overall, this experiment showed that the PPC control samples were not free of aflatoxins, but that the concentrations were low. Additionally, aflatoxins seemed to be heterogeneously distributed throughout the contaminated peanut cake samples. In the current study a full recovery of the initial aflatoxin mass is shown, and AFB_1 metabolism occurred. Most of the aflatoxins were found back in the residual material whereas a low concentration was detected in the larval body. It seems that most aflatoxins are excreted and detoxification activity seems limited. Even though grown on highly contaminated substrates, the concentrations of aflatoxins present in the BSFL were far below the maximum limit of AFB_1 (20 µg/kg) in all feed materials for animal feed mentioned in the EU Directive 2002/32/EC (EC 2002a). This provides a promising

outlook for the safety of rearing insects on aflatoxin contaminated PPC with the purpose of further using them as food- and/or feed. Furthermore, as BSFL weight and survival were not affected, PPC can be considered as a suitable substrate for insect rearing and can be applied globally.

Acknowledgements

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Supplementary materials

Table S1
Instrumental MS/MS parameters of mycotoxins analysed in positive ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
AFB_1 (ql)	4.70	313.1	128.1	40	10	91	10
AFB_1 (qn)	4.70	313.1	285.2	40	10	33	16
AFB_1 (ql2)	4.70	313.1	241.0	40	10	54	15
AFB_2 (ql)	4.40	315.1	259.2	40	10	43	18
AFB_2 (qn)	4.40	315.1	287.2	40	10	37	18
AFG_1 (ql)	4.1	329.0	200.0	40	10	53	12
AFG_1 (qn)	4.1	329.0	243.2	40	10	39	14
AFG_2 (ql)	3.8	331.1	245.2	40	10	43	14
AFG_2 (qn)	3.8	331.1	313.2	40	10	35	18
AFL (qn)	5.40	297.1	269.0	40	10	29	16
AFL (ql)	5.40	297.1	114.9	40	10	81	12
AFL (ql2)	5.40	297.1	141.0	40	10	65	14
AFP_1 (qn)	4.20	299.0	271.0	40	10	33	18
AFP_1 (ql1)	4.20	299.0	114.9	40	10	71	12
AFP_1 (ql2)	4.20	299.0	90.9	40	10	67	10
AFM_1 (qn)	3.84	328.9	272.9	40	10	33	18
AFM_1 (ql)	3.84	328.9	229.0	40	10	55	16
AFQ_1 (qn)	3.70	329.1	310.8	40	10	29	16
AFQ_1 (ql)	3.70	329.1	177.0	40	10	45	22
AFQ_1 (ql2)	3.70	329.1	128.0	40	10	67	14

qn: quantifier ions. ql: qualifier ions

Table S2
Recovery of the mycotoxins in the different matrices (PPC, larvae and residue) analysed

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
PPC	97%	93%	98%	104%	92%	89%	99%	96%
Larvae	86%	84%	93%	85%	91%	88%	91%	90%
Residue	148%	149%	109%	105%	122%	128%	107%	93%

Table S3
LOD and LOQ (ng/mL) per mycotoxin in the different matrices

Compound		AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
PPC	LOD	< 0.2376	0.142	0.081	0.076	0.430	0.297	0.400	0.275
	LOQ	< 0.2376	0.284	0.121	0.381	1.435	0.989	0.889	0.917
Larvae	LOD	< 0.2376	0.157	0.085	0.093	0.435	0.300	0.435	0.293
	LOQ	< 0.2376	0.314	0.128	0.466	1.451	1.000	0.967	0.978
Residue	LOD	< 0.2376	0.089	0.073	0.075	0.325	0.206	0.370	0.284
	LOQ	< 0.2376	0.177	0.109	0.377	1.082	0.688	0.822	0.946

LOD: limit of detection; LOQ: limit of quantification

Table S4
Molecular weight of parent compounds and analysed metabolites for mass calculation purposes

Compound	Molecular weight (g/mol)
AFB_1	312.27
AFB_2	314.29
AFG_1	328.27
AFG_2	330.29
AFL	314.29
AFP_1	298.25
AFM_1	328.27
AFQ_1	328.27

Table S5
Overview and sum of the concentration (µg/kg) of aflatoxins in the peanut cake, larval and residue samples

	Concentration (µg/kg)								
Sample Name	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1	SUM aflatoxins
Peanut cake Control (1)	1.77	0.34	0.44	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	2.55
Peanut cake Control (2)	0.59	< LOQ	0.21	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.80
Peanut cake Control (3)	0.69	< LOQ	0.20	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.89
Peanut cake AFB_1 (1)	9.82	1.80	0.68	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	12.30
Peanut cake AFB_1 (2)	18.06	7.08	0.80	< LOQ	< LOQ	< LOQ	1.60	< LOQ	27.54
Peanut cake AFB_1 (3)	8.64	1.69	2.08	0.74	< LOQ	< LOQ	< LOQ	< LOQ	13.15
Peanut cake AFB_1 (4)	21.64	4.70	1.17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	27.51
Peanut cake AFB_1 (5)	11.82	2.51	1.17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	15.50
Peanut cake AFB_1 (6)	18.00	2.86	0.92	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	21.78
Larvae Control (1)	0.25	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.25
Larvae Control (2)	2.30	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	2.30
Larvae Control (3)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Larvae AFB_1 (1)	0.50	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.50
Larvae AFB_1 (2)	0.80	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.80
Larvae AFB_1 (3)	0.80	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.80
Larvae AFB_1 (4)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Larvae AFB_1 (5)	0.26	< LOQ	< LOQ	< LOQ	<LOQ	< LOQ	< LOQ	< LOQ	0.26
Larvae AFB_1 (6)	0.41	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.41
Residue Control (1)	3.27	0.83	0.37	< LOQ	< LOQ	2.38	< LOQ	< LOQ	6.84
Residue Control (2)	12.57	0.91	0.53	< LOQ	< LOQ	4.07	< LOQ	< LOQ	18.08
Residue Control (3)	1.52	0.44	0.44	< LOQ	< LOQ	2.03	< LOQ	< LOQ	4.43
Residue AFB_1 (1)	13.05	2.48	1.07	< LOQ	< LOQ	2.10	0.84	< LOQ	19.54
Residue AFB_1 (2)	19.36	3.70	0.77	< LOQ	1.63	3.34	1.24	< LOQ	30.04
Residue AFB_1 (3)	10.90	1.95	2.20	< LOQ	1.34	2.14	< LOQ	< LOQ	18.53
Residue AFB_1 (4)	14.74	2.80	0.79	< LOQ	< LOQ	2.43	1.05	< LOQ	21.81
Residue AFB_1 (5)	8.93	1.83	0.64	< LOQ	1.19	1.81	< LOQ	< LOQ	14.40
Residue AFB_1 (6)	20.68	3.83	1.03	< LOQ	< LOQ	2.79	1.02	< LOQ	29.35

< LOQ: below the limit of quantification

Table S6
Overview of the average contribution (n=6) of the aflatoxins to the overall molar mass balance (%)

Aflatoxin	Larvae		Residual material	
	(%)	SD	(%)	SD
AFB_1	0.22	0.17	65	17
AFB_2			12	3
AFG_1			5	5
AFL			3	4
AFM_1			3	2
AFP_1			11	3

Chapter 8

Conversion of mycotoxin-contaminated maize by black soldier fly larvae into feed and fertilizer

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Abstract

Globally, large amounts of various crops such as cereals, oilseeds, nuts and spices are contaminated with mycotoxins during pre-harvest, postharvest handling, processing and/or storage. Mycotoxin contamination results into economic and health issues, and valorisation options of contaminated crops are urgently needed. The aim of this research was to evaluate whether quality feed and fertilizer can be safely produced from naturally mycotoxin-contaminated crops using black soldier fly larvae (BSFL, *Hermetia illucens* L.) under realistic field conditions in East Africa. Naturally mycotoxin-contaminated maize (corn; *Zea mays* L.) was used as a model due its prevalence as food and feed and utilized by BSFL together with local agri-food by-products at a research facility in Rwanda. To assess the influence of the initial maize mycotoxin contamination and maize inclusion, larval diets with three mycotoxin contamination level and two maize inclusion levels were tested. BSFL were tolerant against the high mycotoxin concentrations (*e.g*. 99.4 µg aflatoxin B_1 kg dry mass^{-1}) as the presence of mycotoxins in the substrate did not affect BSFL mass. Product safety was assessed by quantifying the presence of 38 common and emerging mycotoxins and metabolites in the maize, substrates and BSFL products (*e.g*. larvae and frass). The results show that it is possible to produce feed and fertilizer with BSFL considered safe within EU and East African legal limits from maize contaminated with mycotoxin-contaminated maize typical for East Africa. Thereby, this research works towards the safe recycling of nutrients from mycotoxin-contaminated maize within the food system in East Africa and beyond.

Introduction

Mycotoxins are secondary metabolites produced by fungi. Mycotoxins count in hundreds and exposure to them can affect human and animal health (Zain 2011). However, the type of toxicity depends on the chemical structure, the dose and duration of exposure (Bennett and Klich 2003; Chilaka *et al.* 2022). In general, mycotoxins can cause both acute toxicity and chronic carcinogenicity and are known to be carcinogenic, mutagenic, teratogenic, and immunosuppressive amongst others. Furthermore, exposure may lead to gastrointestinal symptoms *e.g.* feed refusal and vomiting which affects productivity in livestock (Fink-Grernmels 1999; Prelusky *et al.* 1994). Amongst the mycotoxins, the aflatoxins are considered as the most toxic human carcinogens (IARC 2012). It is worth noting that there are hundreds of different types of mycotoxins; most concerning are aflatoxins (*e.g*. aflatoxin B_1 (AFB_1)), fumonisins (*e.g*. fumonisin B_1 (FB_1)), zearalenone (ZEN), and deoxynivalenol (DON), mainly produced by *Aspergillus, Fusarium*, and *Penicillium* (Ankwasa *et al.* 2021). Mycotoxin contamination is especially a challenge in tropical countries due to favourable fungi growth conditions (*i.e.* warm and humid environmental conditions), poor crop management practices, and non-existing standard enforcement (Chilaka *et al.* 2022; Matumba *et al.* 2017). In addition, mycotoxin contamination causes economic impact due to low market value of contaminated products, or

their rejection from high-value markets (Adeyeye *et al.* 2022). It is expected that climate change will worsen the current situation (Medina *et al.* 2015). Different management strategies are used to mitigate mycotoxin contamination, along value chain. Pre-harvest measurements include agronomic (*e.g*. planting and harvesting timing, crop rotation), biological (*e.g*. fungal antagonists) and chemical (*i.e.* fungicides and insecticides) means (Conte *et al.* 2020). Post-harvest strategies include a good storage management (*e.g*. control of temperature and humidity control), and biological (*e.g.* bacteria, fungi, enzymes), physical (*e.g*. sorting, drying, thermal inactivation, irradiation) and chemical (*e.g.* ozone, organic acids) methods (Peng *et al.* 2018). Although there are strategies in place, the issue of mycotoxin contamination along crop value chains remains significant. Contaminated batches of crops are regularly present in food systems; therefore, more solutions are needed to manage contaminated crops.

Black soldier fly larvae (BSFL; *Hermetia illucens L*.) have emerged as a promising approach because they can grow on a variety of low value and mycotoxin-contaminated substrates (Camenzuli *et al.* 2018; *Chapter 2*; Gold *et al.* 2018; Niermans *et al.* 2021). Growing BSFL on mycotoxin-contaminated food and by-products is different from using pre- and post-harvest technologies that protect agricultural products from mycotoxin contamination. Instead, BSFL feed on mycotoxin-contaminated products and thereby convert it into nutrient-rich larval biomass and compost-like frass (*i.e.* BSFL excreta and undigested substrate). The larval biomass has particular value as animal feed (*e.g*. pets, poultry, pigs) (Barragan-Fonseca *et al.* 2017) or food but also for biotechnological, cosmetic and pharmaceutical applications. In low-income countries, larval biomass (*e.g.* live, dried or defatted) can increase the availability, quality and affordability of animal feeds important for food security and livelihoods. In addition, the frass remaining from larval bioconversion has value as organic fertilizer (Fuhrmann *et al.* 2022).

Interestingly, research to date has shown that BSFL are highly tolerant and do not bioaccumulate AFB_1, DON, FB_1, OTA and ZEN present in the substrate (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 4*; Leni *et al.* 2019; Niermans *et al.* 2023b; Purschke *et al.* 2017). Only Heuel *et al*. (2023) has observed a slower larval development in comparison to an uncontaminated control after spiking food waste with 800 µg AFB_1 kg DM^{-1}. Similar to the other studies, even at these high concentrations, only small amounts of AFB_1, below legal limits for feed in the EU were found in the larvae and frequently attributed to undigested substrate in the larval digestive tract. Even though considerable amounts of mycotoxins and decomposition metabolites are found in the frass, concentrations are frequently less than in the initial substrate suggesting that similar to other insects, BSFL and associated microbes have mycotoxin metabolization pathways (Evans and Shao 2022; Meijer *et al.* 2019; Suo *et al.* 2023). These results are promising but evidence is lacking on the utilization of naturally contaminated maize in subtropical and tropical regions. To date, most studies on the utilization of mycotoxin-contaminated substrates with BSFL were completed at a laboratory scale, with high quality substrates and/or one or several artificially

spiked mycotoxin contaminations (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Heuel *et al.* 2023). This is a shortcoming considering that in reality, mycotoxins frequently co-occur (Ankwasa *et al.* 2021). In addition, artificially spiking with one or several mycotoxins may produce different results than naturally contaminated materials. Purschke *et al*. (2017) and Leni *et al*. (2019) studied naturally contaminated crops but only at relatively low mycotoxin concentration relative to peak concentrations found in crops in subtropical and tropical regions.

The objective of this study was to assess the viability of producing high-quality feed and fertilizer using BSFL from crops contaminated with mycotoxins, in real-world field conditions in Rwanda, Africa. By doing so, this research aims to promote the safe recycling of nutrients from mycotoxin-contaminated maize in the food system of East Africa and other regions.

Materials and methods

Experimental design and substrates

The study consisted of maize and BSFL substrate analyses, larval feeding trials and post-harvest analyses of products (*i.e.* larvae and frass). Six different substrates (Table 1) were used to test the influence of maize inclusion and substrate maize mycotoxin contamination levels on product mycotoxin concentrations and BSFL growth. Clean maize (4.8 µg kg^{-1} total aflatoxins, AgraStrip Pro Total Aflatoxin WATEX, Rober Labs, Getzendorf, Austria) was mixed with contaminated maize (> 460 µg kg^{-1} total aflatoxins) at increasing ratios starting from the clean maize to produce maize with three mycotoxin contamination levels: control maize (CM, 0% mycotoxin-contaminated maize), low mycotoxin-contaminated maize (LM, 25% mycotoxin-contaminated maize) and high mycotoxin-contaminated maize (HM, 100% mycotoxin-contaminated maize) (Table 1).

Maize with the three mycotoxin-contaminated levels (CM, LM and HM) was then mixed at two levels with local agri-food by-products resulting in 50% and 80% of maize (based on dry mass (DM) of substrate, Table 1). Clean maize was provided by a maize handling company (AflaSight, Kigali, Rwanda) following automatic sorting (SORTEX A LumoVision, Bühler, Uzwil, Switzerland) and unprocessed contaminated maize was purchased from a local food market (Nyabugogo, Kigali, Rwanda). Both clean and contaminated maize were milled to 1.2 mm maize flour (Figure S1) and classified as clean and contaminated based on limits for whole grains in the East African Community (EAC) (10 µg kg DM^{-1}, Table S16). The agri-food by-products (see Figure S2) consisted of homogenized brewers spent grain and fresh fruits stored for four days to mimic typical decomposition of agri-food by-products.

Table 1. Summary of substrates assessed in this study and their proportion of maize and agri-food by-products and clean and contaminated maize

Substrate	Abbreviation	Substrate composition (%DM)		Maize composition (%)		
		Maize	Agri-food by-product	Mycotoxin level	Clean	Contaminated
1	CM50	50%	50%	Control	100%	0%
2	LM50	50%	50%	Low	75%	25%
3	HM50	50%	50%	High	0%	100%
4	CM80	80%	20%	Control	100%	0%
5	LM80	80%	20%	Low	75%	25%
6	HM80	80%	20%	High	0%	100%

DM: dry matter; CM50: with control maize, 50% DM maize; LM50: Low mycotoxin-contaminated maize, 50% DM maize; HM50: high mycotoxin-contaminated maize, 50% DM maize; CM80: with control maize, 80% DM maize; LM80: Low mycotoxin-contaminated maize; 80% DM maize, HM80: high mycotoxin-contaminated maize, 80% DM maize

Black soldier fly larvae feeding trials

Young BSFL from a colony at The Bug Picture at Bishenyi, Rwanda, were reared on pig feed for seven days according to procedures described in the supplementary material to around 0.3 mg $larva^{-1}$. Thereafter, the larvae were separated from the pig feed residue and reared for twelve days in plastic trays (56 cm x 38 cm) containing 6,705 g substrate (70% moisture content, Table S1) and 7,500 larvae (3.5 larvae per cm -2, 74.5 mg (larva x $day)^{-1}$) according to Gold *et al*. (2020). Temperature and relative humidity measured daily (9 am, 1 pm and 5 pm) with a handheld thermometer during the trial are summarized in the Table S2 and S3. Following twelve days, larval mass was determined, and residue and larval samples were collected by manual separation. No specific procedure allowing larvae to empty their gut was applied. Larvae and residue were placed in plastic zip lock bags and stored at -18°C until further analyses.

Substrate and product nutrient and chemical composition

One pooled sample of the four biological replicates for the substrates and larvae were analysed for moisture content and gross nutrient composition (*i.e.* crude protein from total nitrogen, crude fat, ash) and the frass was analysed for moisture content, typical physicochemical (see Table 4) metrics for compost quality and *Aspergillus.* Analyses were done by an external certified laboratory (Cropnuts, Nairobi, Kenya) according to standard procedures (ISO 1999a; ISO 1999b; ISO 2008a; ISO 2008b; ISO 2009; ISO 2022). One soybean and fishmeal sample from Nyabugogo market in Kigali was analysed in parallel to BSFL using the same procedures as a benchmark for the animal feed quality in Rwanda. Crude protein was estimated by multiplying the nitrogen value with sample-specific factors: 5.6 for substrates (based on maize) (Mariotti *et al.* 2008), 4.7

for larvae (Janssen *et al.* 2017), 5.7 for soybean meal (Mariotti *et al.* 2008) and 6.25 for fishmeal. In parallel, the true protein content was also analysed as the sum of all amino acids by external laboratories for the larvae fed on CM50 (Agrolab, Kiel, Germany), and soybean and fishmeal (Groen Agro Control, Delfgauw, Netherlands) according to EC (2009).

Mycotoxin analyses

The maize, substrates, larvae and frass were analysed in quadruplicate for 38 common and emerging mycotoxins and metabolites (Table 2, Table S4) with an in-house method validated according to EC (2019) at Wageningen Food Safety Research. Samples were ground under liquid nitrogen and then stored at -80°C until analysis (EC 2019b). Sample extraction, quantification of the mycotoxin concentration and LC-MS/MS analyses were mostly done according to Camenzuli *et al*. (2018). In the current study a smaller sample mass (1.0 g) of the substrate, larvae and frass samples was used as mentioned in the original method (2.5 g). The used reagents were adjusted accordingly. Mycotoxin concentration in the sample was quantified by means of standard addition, and the mycotoxin concentrations were analysed with LC-MS/MS. A detailed description of the extraction procedure, method of quantification, LC-MS/MS analyses and the parameters used can be found in the supplementary material.

Data analyses

LC-MS/MS data was analysed with SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA USA). Peak identification was performed according to criteria set in an in-house validated method at Wageningen Food Safety Research and identification and recovery criteria outlined (EC 2019b). A detailed description of the methods utilized to determine a limit of detection (LOD) and limit of quantification (LOQ) of the LC-MS/MS method, final LOD and LOQ concentrations, as well as the formula used for quantification of the mycotoxin concentration in the samples can be found in the supplementary material (Table S5 to S8). For statistical analyses all mycotoxins below the LOQ were considered to be of lower analysis quality and set to 0. Mycotoxin concentrations were analysed together with the maize, substrate and frass compositional data, larval mass as well as substrate/frass temperature from feeding trials in R software and RStudio version 3.6.3 (RStudio Inc., Boston, MA, USA). Mean and standard deviation of results between replicates were calculated. Significance of differences in mean results was assessed using analysis of variance (ANOVA) followed by pairwise Tukey post-hoc comparisons. A p-value of < 0.05 was chosen to declare significance.

Results

Maize and substrates

The contaminated maize had 16 mycotoxins (Table 2), was significantly higher in AFB_1, FB_1 and NPA and included five additional mycotoxins (AFG_2, AFM_1, ZEN, AOH, AC) than the clean maize. In the clean maize relatively small amounts of 13 mycotoxins were detected, whereas the concentration of 15AcDON was significantly higher than that found in the contaminated maize.

The substrates used in the BSFL feeding trials had a moisture content of 65-80%, *i.e.* in the typical range of BSFL substrates. The substrate was low in protein (8-9 %DM), fat (5 %DM), ash (2-3 %DM) and crude fibre (3% DM) with similar results regardless of the maize inclusion level (Table S9).

As expected, mycotoxins found in the substrates were generally those present in the clean and contaminated maize (Table 2). Only, ENNB, $ENNB_1$ and AME were found in small amounts in one or more of the substrates but not in the clean and contaminated maize. AFM_1, 15AcDON and AC were found in very low concentrations in the maize but not in the substrates. Similarly, as expected, substrates formulated with contaminated maize were rich in mycotoxins and the composition of the substrate changed the mycotoxin concentrations in the substrate. Inclusion of a higher percentage of maize with a higher mycotoxin contamination increased the mycotoxin concentrations in the substrate. Overall, aflatoxins (AFB_1, AFB_2, AFG_1) and fumonisins (FB_1, FB_2, FB_3) were significantly higher in LM80 than LM50 and HM80 than HM50 (Table 2). Substrates with clean maize had low mycotoxin concentrations (Table 2) with significantly higher concentration for CM80 than CM50. CM50 and CM80 had significantly lower mycotoxin concentrations than LM50 and LM80, respectively. As expected, concentration of mycotoxins (AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, BEA, MON and NPA) were highest in HM80 as compared to all other substrates (Table 2, Table S10 for statistical analysis). HM80 had 123 ± 17 µg kg DM^{-1} total aflatoxins (AFB_1+AFB_2+AFG_1+AFG_2), 135 ± 17 µg kg DM^{-1} DON and 1,622 ± 251 µg kg DM^{-1} total fumonisins (FB_1+FB_2+FB_3). ZEN was only detected in HM80 and no other substrate.

Influence of maize inclusion and mycotoxin contamination on larval mass

The mean larval mass of CM50, LM50 and HM50 were not significantly different. Larval mass was also not significantly different between CM80, LM80 and HM80 (Figure 1a). However, these conclusions should be interpreted with caution due to the limited data per replicate (n=4) and relatively high standard deviation of the results, and it is plausible that increasing the number of replicates and reducing the standard deviation could reveal significant differences in performance. In contrast, it could be carefully concluded that the maize inclusion level influenced larval growth (Figure 1b). Larval mass was significantly lower on the substrate containing more maize when considering all maize mycotoxin levels (*i.e.* CM80, LM80 and LM80

vs. CM50, LM50 and HM50), however the differences were small. Mean larval mass ranged from 72.8-108.0 mg with 50% DM maize and 72.2-75.8 mg with 80%DM in the substrates. Substrate/frass temperature appeared to be similar between treatments, ranging from 21-27°C. Over the entire feeding trial, mean temperature in the substrate/frass was 23-24°C (Table S2 and S3).

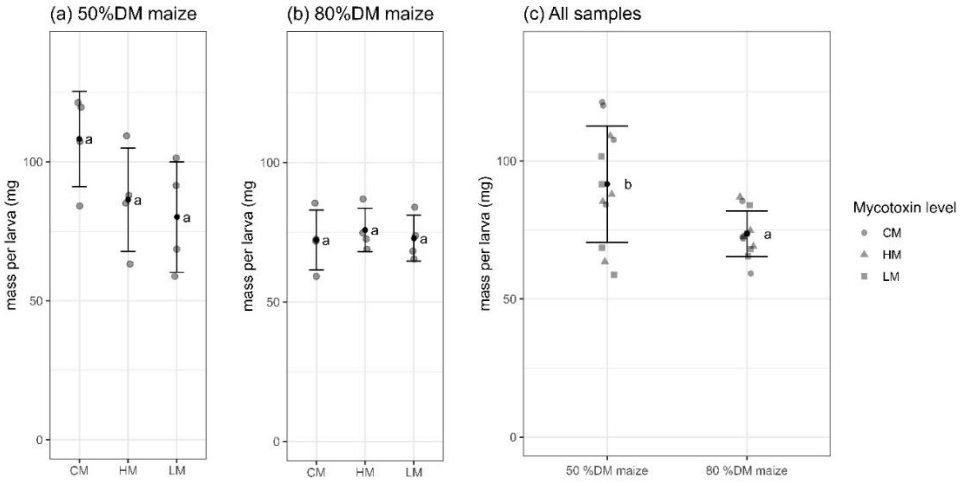

Figure 1. (a, b) Effect of maize mycotoxin contamination on larval mass. (c) Effect of maize inclusion level on larval mass. Means, standard deviations and results per biological replicate are displayed. CM: Control maize, LM: Low mycotoxin-contaminated maize, HM: High mycotoxin-contaminated maize

Mycotoxin transfer from substrate to products

Despite the partially or very high concentration of mycotoxins in the substrate (Table 2), mycotoxins were either only found in low amounts or not detected in BSFL (Table 3). As expected, in the larvae from clean maize (CM50 and CM80) few mycotoxins (*e.g*. AFG_1, FB_1, FB_2 and AME) were detected in low amounts (Table 3). However, also larvae from LM50 and LM80 were not significantly different from larvae from CM50 and CM80, respectively. Even though mycotoxin concentrations in larvae were generally very low, mycotoxin concentration in BSFL appear to increase with higher substrate mycotoxin contamination. Larvae from the substrate with the highest mycotoxin contamination (HM80, Table 2) had significantly more AFB_1, FB_1, FB_2, FB_3 and BEA than larvae from CM80. Larvae from HM50 contained only significantly higher concentrations of FB_1, FB_2, FB_3 and BEA than larvae from CM50.

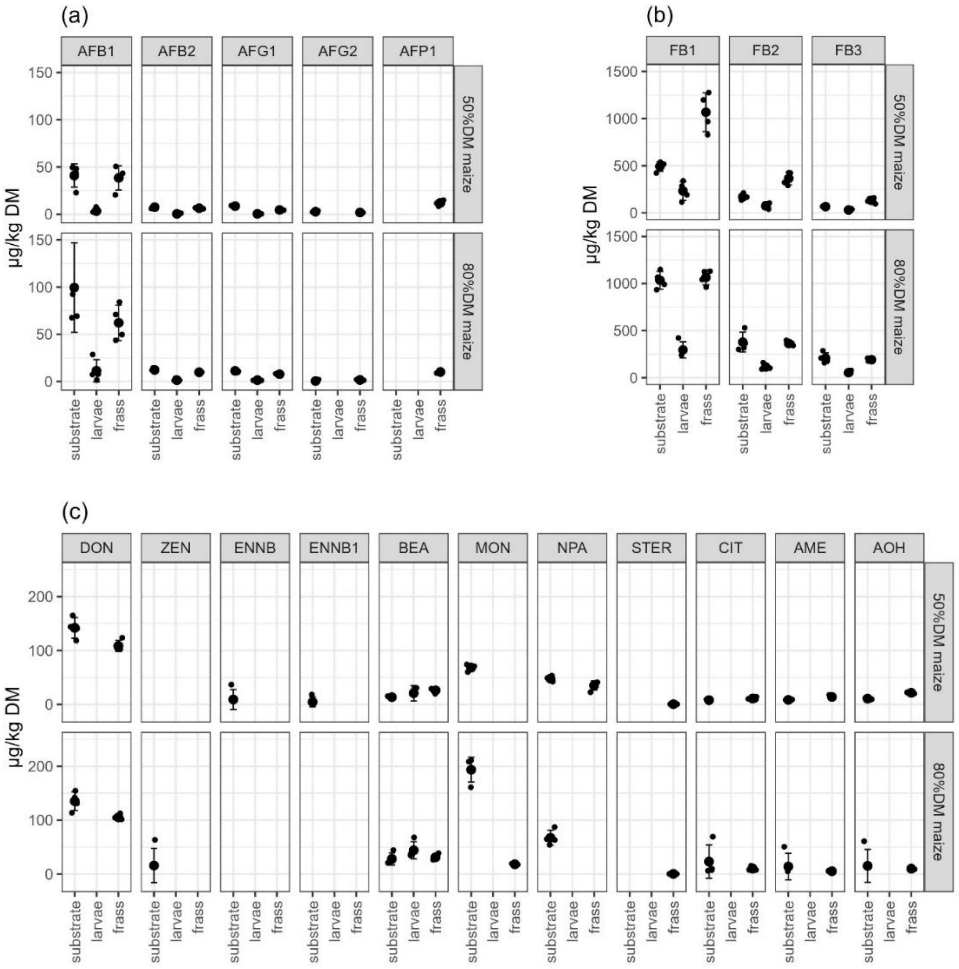

Figure 2. Evolution of mycotoxins between substrate and products (*i.e.* larvae and frass) for the highest contaminated maize inclusion level HM50 and HM80 (n=4). Results < LOQ were set to 0. Significant differences are indicated in Table S13

Table 2. Concentrations of all mycotoxins found in the maize and different substrates (µg kg DM^{-1})

Parameter	Clean maize	Contaminated maize	CM50	LM50	HM50	CM80	LM80	HM80
Aflatoxins[1]								
AFB_1	2.3 (3.2)^a	172.3 (136.7)^b	0.4 (0.5)^a	16.2 (1.7)^b	41.0 (12.4)c	1.5 (0.2)^a	18.8 (3.8)^b	99.4 (47.5)^c
AFB_2	0.2 (0.4)^a	17.5 (19.8)^a	< LOQ^a	2.5 (0.2)^b	7.5 (1.1)^c	< LOQ^a	2.7 (0.1)^b	12.2 (1.4)^c
AFG_1	0.5 (0.5)^a	16.9 (28)^a	0.4 (0.3)^a	3.7 (1.2)^b	8.6 (0.4)^c	1.0 (0.1)^a	3.3 (0.7)^b	11.3 (1.6)^c
AFG_2	< LOQ^a	2.3 (4.2)^a	< LOQ^a	< LOQ^a	2.7 (0.6)^b	< LOQ^a	< LOQ^a	0.5 (1.1)^a
AFM_1	< LOQ^a	1.1 (1.5)^a	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Fusarium mycotoxins[2]								
15AcDON	38.3 (30)^a	< LOQ^b	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
DON	121.2 (33.8)^a	104.7 (50)^a	115.9 (3.6)^a	131.1 (6.3)^{a,b}	142.1 (19)^b	127.6 (7.8)^a	130.7 (7.5)^a	135.2 (17.3)^a
ZEN	< LOQ^a	3.4 (6.8)^a	< LOQ^a	< LOQ^a	< LOQ^a	< LOQ^a	< LOQ^a	15.9 (31.7)^a
FB_1	290 (225.3)^a	2340.4 (1976.1)^b	109.7 (11.1)^a	347.4 (73.3)^b	493.3 (50.3)^c	215.8 (13.5)^a	469.2 (49.2)^b	1034.6 (93.5)^c
FB_2	149.7 (110.2)^a	1446.3 (1780.9)^a	37.8 (8.9)^a	121.8 (34.1)^b	169.2 (31.4)^b	66.8 (5.9)^a	156.3 (30.7)^a	378.6 (104.6)^b
FB_3	35.6 (19.2)^a	208.4 (140.6)^a	12.7 (8.6)^a	45.6 (12.6)^b	65.7 (5.3)^c	29.1 (1.4)^a	69.9 (9.2)^a	208.5 (55.9)^b
ENNB	< LOQ	< LOQ	< LOQ^a	< LOQ^a	9.2 (18.4)^a	< LOQ^a	< LOQ^a	< LOQ^a
$ENNB_1$	< LOQ	< LOQ	< LOQ^a	< LOQ^a	4.6 (9.3)^a	< LOQ^a	< LOQ^a	< LOQ^a
BEA	6.9 (2.5)^a	69.9 (53.4)^a	< LOQ^a	3.0 (5.9)^a	13.5 (1.4)^b	< LOQ^a	3.7 (7.3)^a	28.1 (11)^b
MON	62.1 (33.1)^a	26.5 (52.9)^a	36.1 (4.4)^a	54.9 (8.4)^b	68.3 (5.9)^c	54.6 (3.7)^a	79.2 (12.9)^a	193.5 (23.1)^b
Aspergillus, Penicillium, and Alternaria mycotoxins[3]								
NPA	20 (28.4)^a	101.2 (15.2)^b	< LOQ^a	< LOQ^a	47.8 (4.6)^b	< LOQ^a	< LOQ^a	67.4 (13.9)^b
STER	2.1 (3.9)^a	4.7 (1.4)^a	< LOQ^a	0.2 (0.5)^a	< LOQ^a	< LOQ^a	< LOQ^a	< LOQ^a
CIT	2.1 (3.9)^a	4.7 (1.4)^a	< LOQ^a	0.9 (1.9)^a	8 (1.9)^b	< LOQ^a	0.9 (1.9)^a	23.2 (30.8)^a
AME	< LOQ	< LOQ	9.9 (0.9)^a	12 (1)^b	8.4 (0.6)^a	4.4 (5.3)^a	< LOQ^a	13.9 (24.6)^a
AOH	< LOQ^a	1.0 (2)^a	12 (0.9)^{a,b}	13.9 (1.8)^b	10.7 (1.4)^a	2.2 (4.3)^a	< LOQ^a	15.2 (30.5)^a
AC	< LOQ^a	0.1 (0.2)^a	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Average and standard deviation in parentheses with n=4. Results with no shared letter are significantly different from each other (comparing CM50, LM50, HM50 and CM80, LM80 and HM80 separately). LOQs see Table S8. DM: dry matter. CM50: with control maize, 50% DM maize; LM50: Low mycotoxin-contaminated maize, 50% DM maize; HM50: high mycotoxin-contaminated maize, 50% DM maize. CM80: with control maize, 80% DM maize; LM80: Low mycotoxin-contaminated maize; 80% DM maize, HM80: high mycotoxin-contaminated maize, 80% DM maize. [1]aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and aflatoxin M_1 (AFM_1) . [2]15-acetyldeoxynivalenol (15AcDON), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), beauvericin (BEA) and moniliformin (MON). [3]nitropropionicacid (NPA), sterigmatocystin (STER), citrinin (CIT), alternariol-monomethylether (AME), alternariol (AOH) and agroclavine (AC).

In summary, larvae reared on HM50 and HM80 are the most relevant regarding BSFL product safety and shown in Figure 2 to illustrate the transfer and potential bioaccumulation of mycotoxins from substrates to BSFL products. Except for BEA, mycotoxins in the BSFL were much lower than in the substrates or even absent confirming that no bioaccumulation took place. BEA was the only mycotoxin that bioaccumulated with a factor of 1.6 (concentration BEA in larvae/concentration BEA in substrate) for HM50 and HM80 reared BSFL (Figure 2b). In contrast to the larvae, aflatoxins, DON, fumonisins, BEA, CIT, AME and AOH were detected in the frass (Figure 2). Frass mycotoxin concentrations relative to the initial substrate mycotoxin concentration varied among substrates (0-3.9, frass concentration/substrate concentration) and mycotoxins (Figure 2, Table S11 to S13). Most often, frass mycotoxins were significantly lower or not significantly different from the substrate. For example, AFB_1 in frass from HM50 and HM80 was significantly lower than AFB_1 in the substrate but not significantly different between frass and substrate for DON. However, frass mycotoxins could also be significantly higher from the substrate. For example, frass from HM50 was significantly higher in FB_1, FB_2, FB_3, STER, AME and AOH than the substrate. Interestingly AFP_1, a known metabolite of AFB_1, was only found in the frass, but not the larvae and substrate, indicating that metabolism of AFB_1 occurred (Figure 2a).

Table 3. Mycotoxins in unprocessed larvae from different substrates containing different amounts of mycotoxin-contaminated maize and maize (µg kg DM^{-1})

Parameter	CM50	LM50	HM50	CM80	LM80	HM80
AFB_1	< LOQ[a]	< LOQ[a]	3.4 (3.2)[a,b]	< LOQ[a]	3.61 (1.2)[a,b]	11.4 (11.8)[b]
AFB_2	< LOQ[a]	< LOQ[a]	0.4 (0.6)[a]	< LOQ[a]	0.4 (0.3)[a]	1.5 (1.5)[a]
AFG_1	< LOQ[a]	0.1 (0.2)[a]	0.4 (0.7)[a]	0.3 (0.3)[a]	0.3 (0.4)[a]	1.5 (1.4)[a]
FB_1	46.8 (9.15)[a]	84.9 (13.4)[a]	232.7 (99.7)[b]	41.6 (31.0)[a]	99.3 (30.6)[a]	296.0 (85.2)[b]
FB_2	15.3 (2.58)[a]	28.6 (4.9)[a]	73.5 (30.8)[b,c]	15.9 (11.5)[a]	37.6 (7.4)[a,b]	114.3 (31.1)[c]
FB_3	< LOQ[a]	3.5 (7.0)[a]	30.2 (12.4)[b]	< LOQ[a]	15.3 (3.5)[a,b]	54.6 (16.2)[c]
BEA	< LOQ[a]	< LOQ[a]	20.9 (14.3)[b]	< LOQ[a]	< LOQ[a]	44.2 (15.8)[c]
AME	1.0 (2.1)[a]	< LOQ[a]	< LOQ[a]	< LOQ[a]	< LOQ[a]	< LOQ[a]

Results with no shared letter are significantly different from each other (comparing CM50, LM50, HM50 and CM80, LM80 and HM80 separately). CM50: with control maize, 50% DM maize; LM50: Low mycotoxin-contaminated maize, 50% DM maize; HM50: high mycotoxin-contaminated maize, 50% DM maize. CM80: with control maize, 80% DM maize; LM80: Low mycotoxin-contaminated maize; 80% DM maize, HM80: high mycotoxin-contaminated maize, 80% DM maize. Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), beauvericin (BEA) and alternariol-monomethylether (AME).

Product composition

BSFL from substrates with the two maize inclusion levels were low in ash (4 %DM) and had 26-29 %DM crude protein and 23 %DM crude fat (Table S14). BSFL from 50 %DM maize had 26.1 %DM true protein confirming that an appropriate nitrogen to crude protein conversion factor

was used. BSFL had 13-16 %DM more crude protein, while having less crude fibre and a similar ash content than soybean meal. Fishmeal had 13-16 %DM more crude protein than BSFL. Soybean meal and fishmeal had 10.2 %DM and 45.0 %DM true protein, respectively. *Aspergillus spp.* was not detected in any of the frass samples. The frass was high in nitrogen, phosphorus, potassium, and carbon (Table 4). Noteworthy, frass from both maize inclusion levels had an overall high electrical conductivity (EC) (4.0 and 6.0 mS cm^{-1}) and pH was much higher with 50% than 80 %DM maize.

Discussion

Mycotoxin contamination of crops is a global problem with enormous health and economic impacts. Particularly in tropical and subtropical low-income countries where crops are indispensable for food security and livelihoods, new approaches are needed to valorise mycotoxin-contaminated crops. Given the economic importance of crops in low-income countries, these approaches do not only increase food security and safety but provide economic value to farmers producing mycotoxin-contaminated crops. Such economic value could come from producing feed and fertilizer from mycotoxin-contaminated crops with BSFL.

The mycotoxin-contaminated maize used in this study was representative for mycotoxin-contaminated maize in Rwanda, or elsewhere. The contaminated maize was sourced from a public food market in Kigali, heavily contaminated with various mycotoxins confirming that mycotoxins co-occur (Ankwasa *et al.* 2021). Maize was unsafe as food and feed based on EAC, and EU legal limits/guiding values (5 µg AFB_1 kg^{-1}, 75 µg DON kg^{-1}, 75 µg ZEN kg^{-1}, 3 µg OTA kg^{-1}, 2,000 µg fumonisins kg^{-1}) (EC 2002a; EC 2006b); Table S16). The contaminated maize in this study (172 µg AFB_1 kg DM^{-1}, 3,995 µg fumonisins kg DM^{-1}) exceeded EAC legal limits for food by 17-times for AFB_1 (5 µg kg^{-1}) and 2-times for fumonisins (2,000 µg kg^{-1}) (Table S16). AFB_1 exceeded the EAC limits as feed (20 µg kg^{-1}) while fumonisins, DON, ZEN and OTA were below the EU guiding values for maize as feed (Table S16). AFB_1 and fumonisin concentrations found in this study were in the range as earlier found in Rwanda (0-500 µg kg^{-1} total aflatoxins and 6,000 µg kg^{-1} fumonisins) (Nishimwe *et al.* 2017). These results highlight the potential risks associated with mycotoxin-contaminated maize entering the food chain as food and/or feed, the potential for acute or toxic impacts to humans or animals, and the urge to find viable solutions such as recycling into feed and fertilizer by BSFL. The clean maize after sorting-out of aflatoxin-contaminated maize had AFB_1 and fumonisins concentrations below EAC and EU limits.

As anticipated, formulating the mycotoxin-contaminated maize with agri-food by-products, presumably absent or low in mycotoxins, resulted in a decrease in mycotoxin concentrations in the substrate. This study aimed to utilize maize that is naturally heavily contaminated with AFB_1, as other studies have only achieved high AFB_1 concentration (400-800 µg/kg DM) by spiking. In comparison to previous studies, the HM80 substrate (Table 2) had the highest AFB_1 (99.4 µg kg

DM^{-1}) and FB_1 concentration (1,035 µg kg DM^{-1}) in naturally contaminated substrates used with BSFL so far (13.3 µg AFB_1 kg DM^{-1}, 727 µg FB_1 kg DM^{-1}). DON, ZEN and OTA were lower in this study in comparison to previous work that used naturally contaminated maize and by-products (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Heuel *et al.* 2023) (Table S15).

Table 4. Physicochemical frass characteristics in comparison to compost and literature values (% DM)

Parameter	Unit	Frass (this study)		Fertilizer Standard Rwanda[1]	Compost Rwanda[2]	Frass Typical range[3]
		50% DM maize	80% DM maize			
DM	%	41.6 (3.6)	43.9 (0.7)	≥ 70	63.4	-
EC	mS cm^{-1}	4.0 (0.09)	6.0 (0.15)	< 5.0	772	1.2-5-7[3,5]
pH	-	8.0 (0.1)	5.9 (1.4)	6-9	7.5	5.4-9.0[4]
N	g kg^{-1}	18.6 (0.2)	20 (0.3)	> 10	15.0 (1.1)	18-51[4]
C	g kg^{-1}	517 (0)	527.7 (1.2)		173.5 (9.5)	268-488[4]
C:N ratio	-	27.8 (0.3)	26.4 (0.3)	≤ 20:1	11.6 (0.3)	7-27[4]
P	g kg^{-1}	1.9 (0.2)	2.3 (0.2)	-	2.4 (0.2)	3-52[4]
K	g kg^{-1}	13.4 (0.5)	6.6 (0.5)	-	3.0 (0.2)	2-41[4]
Ca	g kg^{-1}	< 5.0	< 5.0	> 10	23.7 (3.8)	0.2-45[4]
Mg	g kg^{-1}	1.3 (0.1)	1.1 (0.1)	> 5.0	3.2 (0.5)	0.2-11[4]
Na	mg kg^{-1}	15.8 (7.8)	17.6 (6.5)	-	0.5 (0.0)	0.3-5.0[4]
Fe	mg kg^{-1}	590 (99.9)	337 (112)	1,000-2,500	21.0 (6.4)	4-896[4]
Mn	mg kg^{-1}	22.4 (1)	12.8 (2.1)	200-800	0.9 (0.1)	0.2-149[4]
Zn	mg kg^{-1}	12.7 (1.6)	18.7 (4)	40-1000	0.1 (0.0)	0.1-182[4]
Cu	mg kg^{-1}	3.9 (0.1)	2.3 (0.7)	8-300	28.6 (5.2)	0.7-46[4]

[1]RSB (2021), [2]Fuhrmann *et al.* (2022), [3]Lopes *et al.* (2022). DM: Dry mass, EC: Electric conductivity, N: total nitrogen, C: total carbon, P: total phosphorus, K: Total potassium, Ca: total calcium, Mg: total magnesium, Na: Total sodium, Fe: total iron, Mn: total manganese, Zn: Total zinc, Cu: total copper.

Consistent with previous research (Table S15), BSFL exhibited tolerance to substrate mycotoxins. To date, only Heuel *et al*. (2023) has observed a slower BSFL development with food waste containing 842 µg AFB_1 kg^{-1} DM. In contrast to substrate mycotoxins, our study found that substrate composition (*i.e.* amount of maize and agri-food by-products) significantly influenced larval growth. Greater inclusion of agri-food by-products increased larval mass, likely due to changes in nutrient digestibility that affected larval growth, even though gross nutrients remained similar (Gold *et al.* 2020). Purschke *et al*. (2017) obtained a similar larval mass (75 mg $larva^{-1}$) using a maize-based substrate. However, overall, larval mass in this study was low (73-108 mg larva-1) in comparison to high-performing insect substrates such as restaurant food waste (200 mg $larva^{-1}$). This could be due to low substrate protein (8-9% DM) and fat contents fat (5% DM) crucial for larval development in comparison to higher performing food wastes (*e.g*.

12-32 %DM protein, 29-35 %DM fat, Gold *et al*. 2020). Larval mass could potentially be further improved by incorporating biowaste with higher protein and lipid content, such as animal manure, food waste, oilseed press cakes, or starch or ethanol facility by-products. Additionally, optimizing context-specific rearing conditions (*e.g*. larval density, ventilation rates, substrate texture) and ambient and substrate/frass temperatures which were relatively low in this study (Harnden and Tomberlin 2016) could result in more efficient bioconversion and larval growth. BSFL were reared using the natural climate in Kigali, suggesting that the results could be transferrable to similar climates across Africa.

This study found that BSFL do not generally accumulate substrate mycotoxins, and that larvae from mycotoxin-contaminated substrates were safe as animal feed considering legal standards. Even larvae from substrates with the highest mycotoxin concentrations (HM80, Table 3) had AFB_1 (11.4 µg kg DM^{-1}), FB_1 (296 µg kg DM^{-1}), FB_2 (114 µg kg DM^{-1}) and FB_3 (55 µg kg DM^{-1}) concentrations below legal limits for compound animal feeds in the EU (20 µg AFB_1 kg^{-1}, 5,000 µg DON kg^{-1}, 100-500 µg ZEN kg^{-1}, 10 µg OTA kg^{-1}, 5,000-20,000 µg fumonisins kg^{-1}) (EC 2002a; EC 2006b) and dried insect products for animal feeds in the EAC (20 µg AFB_1 kg DM^{-1}, Table S16). Although larvae at the highest substrate mycotoxin contamination had more AFB_1 than permitted in fish, pig and poultry starter feeds in the EAC (10 µg AFB_1 kg DM^{-1}, Table S16), the authors suggest that a starter compound feed with BSFL and clean other feed ingredients would result in a compound feed AFB_1 concentration below the legal limits considering that they typically include less than 30% of a high-protein ingredient. Overall, this suggests that even at high substrate mycotoxin contamination, BSFL and BSFL-based animal feeds are safe for fish, poultry, and pigs considering current legal limits in the EU and EAC. The authors note that there is currently no legislation for BSFL for food production, but assuming that such legislation would have similar values to unprocessed grains (5 µg AFB_1 kg DM^{-1}, 2,000 µg fumonisins kg DM^{-1}, Table S16), BSFL from all substrates except for HM80 were below legal limits. This suggests that BSFL could be raised for food even on substrates contaminated with mycotoxins up to a certain level of contamination, if BSFL are to be considered as a viable food source in the future (Bessa *et al.* 2020). However, our data confirm that metabolism of AFB_1 took place, likely via the larval cytochrome P450 enzyme system (Meijer *et al.* 2019). It is not fully elucidated if other (unknown) toxic metabolites of mycotoxins could be formed in BSFL which should be further studied to ensure food and feed safety.

Our study confirms previous findings that AFB_1, DON, ZEN, OTA and fumonisins do not bioaccumulate in BSFL, with these mycotoxins typically being below the LOQ (Table S15). However, in studies with higher substrate mycotoxins concentrations (Camenzuli *et al.* 2018; Heuel *et al.* 2023), AFB_1 (4.3 µg kg DM^{-1}), DON (275 µg kg DM^{-1}), ZEN (27.5 µg kg DM^{-1}) and OTA (3.9 µg kg DM^{-1}) concentration in BSFL above the LOQ were reported. Even though BSFL mycotoxin contamination levels are low, substrates with a higher mycotoxin concentration tend

to result in BSFL with higher mycotoxin concentrations. This could also be due to small amounts of undigested substrate in the larval digestive tract. Even though these minor contaminations still make BSFL safe as feed according to legal limits, future research should investigate if they can be removed with egestion approaches of substrates from the BSFL digestive tract. This study is the first to identify bioaccumulation of BEA, with low amounts being detected in BSFL. High contaminated substrates with BEA could potentially result in high concentration of BEA in larvae. This could have caused by the lipophilic nature of this mycotoxin (Tran *et al.* 2020a), potentially accumulating in the larval biomass that is rich in fat.

Frass from mycotoxin-contaminated substrates were high in mycotoxins. However, this does not appear to limit its application as fertilizer as mycotoxins are not regulated in compost and fertilizer (Table S16). However, considering that mycotoxins in frass were partially similar or above the initial high substrate values, post-processing (*e.g*. composting, mixing with compost or fertilizer) and appropriate application and management practices should be considered to prevent negative health impacts following ingestion or inhalation. The contaminated maize and BSFL substrates were rich in aflatoxins produced by the mycotoxin producing fungus *Aspergillus*, suggesting that they contained this import mycotoxin production group of fungi (not quantified). However, no *Aspergillus* were detected in frass with mycotoxin-contaminated substrate suggesting that at least this group of mycotoxin-producing fungi are not propagated to soil when frass is used as fertilizer. Mycotoxins in the frass can help draw conclusion on whether they were decomposed by BSFL and/or the associated microbiome. Because BSFL and microbes reduce the substrate/frass mass during bioconversion (not quantified in this study), a lower or not significantly different frass concentration relative to the initial substrate suggests that mycotoxins were metabolized in the process. Consequently, in this study some part of the aflatoxins and DON were metabolized by BSFL and/or associated microbes. This is similar to previous studies who found 11-18 % AFB_1 and 39-55 % of initial DON in the substrate in larvae and frass (Camenzuli *et al.* 2018). AFP_1, a typical AFB_1 metabolite, was detected in frass and confirms that metabolism took place as mentioned above. In contrast, no conclusions can be drawn on other mycotoxins such as fumonisins because concentration in frass were significantly higher in the frass than the substrate. Previous studies with spiked and naturally contaminated wheat bran and olive pomace have found that 70% of total fumonisins are found in larvae and frass (Leni *et al.* 2019; *Chapter 4*; Niermans *et al.* 2023b). Different trends, meaning that some mycotoxins in frass were below and some above initial concentrations in the substrate, suggest that metabolization during the bioconversion process could vary among mycotoxins. Suo *et al*. (2023) recently concluded for AFB_1, that such decomposition is partially mediated by the digestive tract microbiome which should be investigated for other mycotoxins.

Our study suggests that the frass from maize and agri-food by-products has potential as compost and/or fertilizer. Frass nutrients were comparable to a Rwandan compost (Fuhrmann *et al.*

2022)(Table 4), indicating that frass has a similar fertilizer value. Frass contained 19-20 N, 1.9-2.3 P and 7-13 K (mg kg DM^{-1}), whereas compost had 15 N, 2.4 P and 3 K (mg kg DM^{-1}). Frass contained 3-times more carbon than the compost (520-530 *vs.* 173 mg kg DM^{-1}). Frass varies greatly among substrates, particularly in terms of P, K and micronutrients (Lopes *et al.* 2022), but the frass fell within the typical range (Table 4). Our frass was higher in EC (4-6 *vs.* 1.2-5.7 mS cm^{-1}), C (517-528 *vs.* 268-488 mg kg DM^{-1}), C:N (26-28 *vs*. 7-24 mg kg DM^{-1}) and Na (16-18 *vs.* 0.3-5.0 mg kg DM^{-1}) than the frass reviewed by Lopes (2022). Future research should determine whether the high frass EC is due to ammonia produced by BSFL excretions, which could be a valuable nutrient for plants, or related to salt, which could be toxic to plants, especially those that are salt-sensitive (Beesigamukama *et al.* 2021; Liu *et al.* 2019b). This research should also measure soluble nutrients (*e.g*. P2O5-K2O), assess optimal plant applications (*e.g*. soil and plant type, dose), and investigate nutrient supplementation to meet crop-specific nutrient requirements and fertilizer standards in Rwanda (Table 4) (Table S16) where the frass from our study falls short in terms of EC, C:N ratio, and some micronutrients (Ca, Fe, Mn, Zn, Cu). As highlighted previously, such plant trials with frass could also reveal effects of additional frass compounds, such as bioactive compounds (*e.g*. hemic substances, phytohormones, chitin), and beneficial microorganisms that could promote high plant health and yield (Fuhrmann *et al.* 2022; Lopes *et al.* 2022).

The unprocessed BSFL appear to have value as feed and food due to their protein and fat content. However, the protein content of the larvae (26-29% DM) was lower than the typical range for BSFL (32-58% DM), while fat content (23% DM) fell within the typical range (18-39% DM) (Gold *et al.* 2018). The larvae's protein and fat content is influenced by substrate composition and operational parameters, such as temperature, humidity, air flow, larval densities, and feeding rate. Future research should focus on optimizing these parameters to increase larval protein content and thereby improve the economics of using BSFL to manage mycotoxin-contaminated by-products such as maize. While the larval protein content was below average, unprocessed larvae from our substrates including mycotoxin-contaminated maize had twice as much protein (26-29% DM) than soybean meal (13% DM) in Rwanda. After optimizing substrate and bioconversion conditions, BSFL could be competitive with soybean and fishmeal. Removing fat from BSFL with an oil press can increase protein content up to 59% DM (Maurer *et al.* 2016). This would also be required to make the BSFL competitive with high-quality soybean and fishmeal other than those analysed in this study. Future research should include feeding trials with target animals to optimize larval product quality, considering ash and crude fibre contents, which are typically considered undesirable and indigestible by livestock. BSFL had similar ash (4% DM) and lower fiber content (16-17% DM) than soybean meal, and lower ash than fishmeal (4% DM *vs.* 21% DM). Only fishmeal had a lower fiber content than BSFL (6% DM *vs*. 16-17% DM), possibly due to chitin in the larval exoskeleton.

Conclusions

BSFL can be used to produce feed and fertilizer within legally safe limits from maize contaminated with mycotoxins at concentrations typical for African. Mycotoxins in the substrate did not affect BSFL process performance and mycotoxins in BSFL were found in low amounts, not detected and below the legal limits in the EU and EAC. BSFL grown on contaminated maize had a superior feed value as compared to locally obtained soybean meal. Frass was absent in *Aspergillus* and rich in plant nutrients. Future research should elucidate if unknown toxic metabolites of mycotoxins could be formed in BSFL and optimize the substrate composition and BSFL production system to maximize feed and fertilizer production. Given that BSFL amounts that can be included in feed are a lot lower than the initial crops, pre- and post-harvest strategies to prevent and minimize crop mycotoxin contamination should be prioritized before utilization by BSFL.

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Supplementary Materials

Supplementary Information

Materials and methods

Rearing feedstocks

Experimental design and substrates

- Maize was milled (ring sieve pore size 1.2 mm) using a disk mill (model FFC-15, Qingdao RuiDa Machinery Co., Ltd., Qingdao, China)
- Spent grain (approximately 20% barley, 80% sorghum, based on wet mass) was obtained from a brewery (Kweza Craft Brewery, Kigali, Rwanda).
- Fruits were green banana (45%), pineapple (23%), tree tomato (10%), avocado (9%), and yellow banana (9%) from Nyabugogo market (Kigali, Rwanda, all values based on wet mass).

Black soldier fly larvae feeding trials

The black soldier fly eggs were from a colony at The Bug Picture at Bishenyi, Rwanda. Eggs were removed from the egg media and placed for 24 h on pig feed (40% moisture content). Following, eggs were removed, and neonates were allowed to feed for seven days to around 0.3 mg larva^{-1}.

Substrate and larvae composition

Moisture content was determined as the gravimetric loss while drying at 103°C until constant mass loss. Total nitrogen was determined by combustion and the substrate. Crude fat was estimated by extraction with petroleum ether after hydrolysis using hydrochloric acid. Crude fibre of larvae, soybean and fishmeal was estimated as the gravimetric loss during treatment with boiling sulphuric acid and sodium hydroxide. Ash was determined based on the gravimetric loss during combustion at 550°C for at least 3 h.

- International Organization for Standardization;, 2022. ISO 5984. Animal feeding stuffs-Determination of crude ash.
- International Organization for Standardization;, 2009. ISO 5983. Animal feeding stuffs-Determination of nitrogen content and calculation of crude protein content.
- International Organization for Standardization;, 2008a. ISO 13906. Animal feeding stuffs-Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents.

- International Organization for Standardization;, 1999a. ISO 6492. Animal feeding stuffs-Determination of fat content.
- International Organization for Standardization;, 1999b. ISO 6496. Animal Feeding Stuffs-Determination of Moisture and Other Volatile Matter Content.

Frass composition

Electric conductivity (EC) and pH with a handheld EC and pH meter. Total carbon (C) and nitrogen (N) was determined by combustion, and phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) was determined with ICP-OES following nitric and hydrochloric acid digestion. *Aspergillus spp.* were enumerated by plate counts on selective media. All physicochemical metrics except for C and N were determined with US EPA methods (see supplementary material).

- Association of Official Analytical Chemists (AOAC), 1997. Microchemical Determination of Carbon, Hydrogen, and Nitrogen, Automated Method, in: Official Methods of Analysis of AOAC International. Arlington VA, USA.
- United States Environmental Protection Agency (EPA), 2004. Method 9045D: Soil and Waste pH. Washington DC, USA.
- United States Environmental Protection Agency (EPA), 1996. Method 9050A: Specific Conductance. Washington DC, USA.
- United States Environmental Protection Agency (EPA), 1994. Method 200.7: Determination of Metals and Trace Elements in Water and Wastes by Inductively Coupled Plasma-Atomic Emission Spectrometry. Cincinnati OH, USA.
- International Organization for Standardization;, 2008b. ISO 21527-2: Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 2: Colony count technique in products with water activity less than or equal to 0,95

Mycotoxin analyses

Sample preparation, extraction and analysis

Mycotoxin standards were purchased from BioAustralis: enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$); CoringSystem DiagnostiX: alternariol-monomethylether (AME), alternariol (AOH), agroclavine (AC), diacetoxyscirpenol (DAS), deoxynivalenol-3-glucoside (DON3G), fumonisin B_3 (FB_3), HT-2 toxin (HT-2), mycophenolic acid (MPA), nivalenol (NIV), penicillic acid (PA), roquefortine C (ROQ), sterigmatocystin (STER), T-2 toxin (T-2); Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL); Romer Labs (Getzersdorf, Austria): aflatoxin M_1 (AFM_1); Sigma-Aldrich: 15-acetyldeoxynivalenol (15AcDON), 3-acetyldeoxynivalenol (3AcDON),

aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), beauvericin (BEA), citrinin (CIT), deoxynivalenol (DON), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), moniliformin (MON), nitropropionic acid (NPA), ochratoxin A (OTA), α-zearalenol (α-ZEL), zearalenone (ZEN), β-zearalenol (β-ZEL) and TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1). These standards were used to prepare an aflatoxin standard mix (AFP_1, AFQ_1, AFL, AFM_1), and a mycotoxin standard mix (all other mycotoxins as well as AFB_1, AFB_2, AFG_1 and AFG_2) and were utilized for quantification purposes.

Extraction was similar among the samples apart from a smaller sample mass being used for the substrate, larvae and frass samples (1.0 g) than the maize samples (2.5 g). The used reagents were adjusted accordingly. For the maize, 2.5 g of ground sample were weighed into a 50 mL tube, after which 7.5 mL water was added. The maize/water mixture was then vortexed and allowed to settle for 15 min. Then, 10 mL of extraction solvent, which consists of 99% acetonitrile (HPLC supra gradient, Biosolve Chimie, Dieuze, France) and 1% acetic acid (Merck KgaA, Darmstadt, Germany), was added, followed by vortexing of the samples and 30 min mixing in a head-over-head shaker (Reax 2, Heidolph Instruments GmbH & Co, Schwabach, Germany). Subsequently, 4 g of magnesium sulphate (VWR Chemicals, Radnor, VS) was added, manually shaken and vortexed for 1 min, after which the samples were centrifuged (Falcon 6-30, MSE, Nuaillé, France) at 3000 rpm for 10 min.

Mycotoxin concentration in the sample was quantified by means of standard addition (see supplementary material). The sample without standard addition was prepared by adding 200 µL of sample extract and 200 µL of water into a syringeless PTFE filter vial (Mini-UniPrep, Whatman, Marlborough, MA). The sample with standard addition was prepared by transferring 200 µL of sample extract from the same sample to a second PTFE filter vial after which 10 µL of mycotoxin standard mix, 10 µL aflatoxin standard mix, and 180 µL water were added. After preparation, the vials were capped, vortexed for 30 sec, and stored for a minimum of 30 min at 4°C. After 30 min, the vials were closed and stored at 4°C until LC-MS/MS analyses.

The mycotoxin concentrations were analysed with LC-MS/MS (Waters Acquity injection and pump system, Milford, MA). Additionally, the AB Sciex QTRAP 6500 triple quad system equipped with an electrospray ionization (ESI) source was operated in positive- and negative mode (LC-MS/MS parameters shown in the supplementary material). LC separation was performed by an Acquity UPLC HSS T3 1.8 µm 100x2.1 mm column (Waters). The composition of the eluents used for the positive and negative ionization mode and the LC eluent gradient for both ionisation modes was the same as reported in Camenzuli *et al*. (2018). The flow rate was 0.4 mL/min, the column temperature was 35°C, and the injection volume 5 µL. The conditions set for ESI were as follows: spray voltage 4.0 kV/-4 kV, temperature, 400°C, Ion Source Gas 1 and 2 were both set at 50 arbitrary units.

Data analyses

The mycotoxin concentration was calculated by dividing the sample area without standard addition by the sample area with standard addition minus the sample area without standard addition, multiplied by the mycotoxin concentration in the mycotoxin/aflatoxin standard mix used for standard addition purposes (see equation below).

$$Concentration = \frac{Sample\ area\ no\ addition}{(Sample\ area\ with\ addition) - Sample\ area\ no\ addition}\ x\ concentration\ in\ standard\ mix$$

To obtain the final sample concentrations, the results were corrected for the determined average recovery of the mycotoxins in the different matrices (see supplementary material) to include for possible uncertainties (extraction efficiency, matrix effect, etc) and was done according to EC (2006a).

In order to determine a limit of detection (LOD) and limit of quantification (LOQ) of the method used, a calibration curve (in extraction solvent) was prepared. The LOD and LOQ were determined as the lowest concentration included in the calibration curve with a signal to noise ratio of ≥ 3 and ≥ 10, respectively. However, for some mycotoxins, the lowest point in the calibration curve complied to all set rules. As these concentrations were far (at least 0.13 times) below the legal limits or guidance values (GV) set for animal feed in the EU (see Table 8) (EC 2002a; EC 2006b), or currently no limits or guiding values exist for these mycotoxins, the lowest point of the calibration curve was considered as the LOQ. Final LOD and LOQ concentrations (see supplementary material) were obtained by correcting the concentrations determined in solvent by average recovery of the mycotoxins in the different matrices (EC 2006a).

Supplementary Materials

Table S1
Amounts (in kg) of uncontaminated maize, contaminated maize, agri-food by-products and tap water mixed to produce the six BSFL rearing substrates with two maize inclusion and three maize mycotoxin levels at around 70% moisture content

Abbreviation	Maize	Maize mycotoxin contamination	Uncontaminated maize	Contaminated maize	Agri-food by-product	Tap water
CM50	50% DM	Control	5.7	0	25.4	1.1
LM50	50% DM	Low	4.3	1.4	25.4	1.1
HM50	50% DM	High	0	5.7	25.4	1.1
CM80	80% DM	Control	9.1	0	10.2	12.9
LM80	80% DM	Low	6.8	2.3	10.2	12.9
HM80	80% DM	High	0	9.1	10.2	12.9

Table S2
Ambient temperature and relative humidity during black soldier fly larvae feeding trials

	Temperature				Relative humidity	
Rearing day	Daytime[1]	Range Daytime[2]	Day maximum[3]	Day minimum[3]	Daytime[1]	Range Daytime[2]
1	30.5	25-34	34.2	17.8	44.9	36-67
2	30.6	27-34	34.6	19.4	48.3	37-63
3	26.5	22-34	33.1	16.6	62.5	39-83
4	21.1	20-23	23.4	19.5	81.4	69-95
5	24.9	18-31	32.6	16.5	65.6	44-91
6	23.4	17-28	31.4	17.5	68.6	44-93
7	30.7	26-34	34.6	17.0	45.1	37-61
8	27.0	22-31	32.0	19.2	57.3	45-79
9	28.6	24-34	34.1	17.9	51.1	35-65
10	27.5	25-31	32.9	18.8	53.4	40-65
11	28.5	24-34	36.2	19.0	53.1	38-70
Mean	27.2	22-31	32.6	18.1	57.4	42-57

[1]mean based on point measurements recorded between 7 am and 5 pm; [2]minimum and maximum of point measurements recorded between 7 am and 5 pm; [3]Overall minimum and maximum over the entire day; [4]Mean of daily means over all rearing days

Table S3
Mean substrate/frass temperature of the different treatments over all rearing days (n=4). Frass/substrate temperature was measured with a digital thermometer at 9 am, 1 pm and 5 pm

Rearing day	CM50	CM80	LM50	LM80	HM50	HM80
1	21.9	21.4	21.4	21.9	21.8	22.0
2	24.2	23.6	23.7	23.8	24.2	24.3
3	23.1	22.7	22.8	23.0	23.5	23.3
4	20.6	20.6	20.6	20.4	20.8	20.8
5	21.8	21.5	21.3	21.7	21.3	21.8
5	22.0	21.4	21.5	21.5	21.7	21.9
7	23.6	23.0	22.6	23.2	22.7	23.4
8	23.9	23.1	23.3	23.3	23.1	23.7
9	24.6	23.1	23.9	23.3	24.1	23.4
10	27.1	25.1	26.1	25.3	26.4	25.5
11	27.1	24.2	26.0	24.2	25.9	24.3
Mean	23.6 (2.1)	22.7 (1.3)	23.0 (1.8)	22.9 (1.4)	23.3 (1.8)	22.7 (1.3)

Table S4
Mycotoxins and metabolites analysed in this study.

Aflatoxins	*Fusarium* mycotoxins	Aspergillus, Penicillium, and Alternaria mycotoxins
aflatoxin B_1 (AFB_1)	15-acetyldeoxynivalenol (15AcDON)	Nitropropionic acid (NPA)
aflatoxin B_2 (AFB_2)	3-acetyldeoxynivalenol (3AcDON)	sterigmatocystin (STER)
aflatoxin G_1 (AFG_1)	diacetoxyscirpenol (DAS)	citrinin (CIT)
aflatoxin G_2 (AFG_2)	deoxynivalenol (DON)	Mycophenolic acid (MPA)
aflatoxicol (AFL)	deoxynivalenol-3-glucoside (DON3G)	ochratoxin A (OTA)
aflatoxin M_1 (AFM_1)	HT-2 toxin (HT-2)	Penicillic acid (PA)
aflatoxin P_1 (AFP_1)	nivalenol (NIV)	roquefortine C (ROQ)
aflatoxin Q_1 (AFQ_1)	T-2 toxin (T-2)	alternariol-monomethylether (AME)
	α-zearalenol (α-ZEL)	alternariol (AOH)
	zearalenone (ZEN)	agroclavine (AC)
	β-zearalenol (β-ZEL)	
	fumonisin B_1 (FB_1)	
	fumonisin B_2 (FB_2)	
	fumonisin B_3 (FB_3)	
	enniatin A (ENNA)	
	enniatin A_1 ($ENNA_1$)	
	enniatin B (ENNB)	
	enniatin B_1 ($ENNB_1$)	
	beauvericin (BEA)	
	moniliformin (MON)	

Table S5
Instrumental MS/MS parameters of mycotoxins analysed in positive ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
15AcDON (ql)	3.3	355.9	321.1	40	10	17	20
15AcDON (qn)	3.3	355.9	137.1	40	10	22	10
AFB_1 (ql)	4.70	313.1	128.1	40	10	91	10
AFB_1 (qn)	4.70	313.1	285.2	40	10	33	16
AFB_1 (ql2)	4.70	313.1	241.0	40	10	54	15
AFB_2 (ql)	4.40	315.1	259.2	40	10	43	18
AFB_2 (qn)	4.40	315.1	287.2	40	10	37	18
AFG_1 (ql)	4.1	329.0	200.0	40	10	53	12
AFG_1 (qn)	4.1	329.0	243.2	40	10	39	14
AFG_2 (ql)	3.8	331.1	245.2	40	10	43	14
AFG_2 (qn)	3.8	331.1	313.2	40	10	35	18
AFL (qn)	5.40	297.1	269.0	40	10	29	16
AFL (ql)	5.40	297.1	114.9	40	10	81	12
AFL (ql2)	5.40	297.1	141.0	40	10	65	14
AFP_1 (qn)	4.20	299.0	271.0	40	10	33	18
AFP_1 (ql1)	4.20	299.0	114.9	40	10	71	12
AFP_1 (ql2)	4.20	299.0	90.9	40	10	67	10
AFM_1 (qn)	3.84	328.9	272.9	40	10	33	18
AFM_1 (ql)	3.84	328.9	229.0	40	10	55	16
AFQ_1 (qn)	3.70	329.1	310.8	40	10	29	16
AFQ_1 (ql)	3.70	329.1	177.0	40	10	45	22
AFQ_1 (ql2)	3.70	329.1	128.0	40	10	67	14
AC (ql)	3.2	239.1	208.2	40	10	27	10
AC (qn)	3.2	239.1	183.2	40	10	27	12
BEA (ql)	8.0	784.4	262.3	30	10	41	13
BEA (qn)	8.0	784.4	244.2	30	10	41	13
DON (ql)	2.7	297	231	30	10	17	15
DON (qn)	2.7	297	249	30	10	15	15
DAS (ql)	4.3	384.2	105.1	40	10	61	7
DAS (qn)	4.3	384.2	307.2	40	10	17	9
ENNA (ql)	8.2	699.4	228	40	10	20	16
ENNA (qn)	8.2	699.4	210.1	40	10	20	14
$ENNA_1$ (ql)	8.0	685.4	228.2	40	10	20	16
$ENNA_1$ (qn)	8.0	685.4	210.1	40	10	20	14
ENNB (ql)	7.7	657.5	214.1	40	10	20	22
ENNB (qn)	7.7	657.5	196.3	40	10	20	14
$ENNB_1$ (ql)	7.9	671.4	210	40	10	20	14
$ENNB_1$ (qn)	7.9	671.4	196	40	10	20	14
FB_1 (ql)	5.4	722.5	352.3	40	10	55	12
FB_1 (qn)	5.4	722.5	334.4	40	10	57	4
FB_2 (ql)	6.2	706.4	318.5	40	10	51	2
FB_2 (qn)	6.2	706.4	336.3	40	10	53	8
FB_3 (ql)	5.9	706.4	318.5	40	10	51	2

FB_3 (qn)	5.9	706.4	336.3	40	10	53	8
HT-2 (ql)	5.1	441.9	215.1	40	10	17	16
HT-2 (qn)	5.1	441.9	263.1	40	10	19	6
MPA (ql)	5.7	338.3	303.2	1	10	19	26
MPA (qn)	5.7	338.3	207	1	10	31	16
OTA (ql)	6.4	404	102	40	10	91	14
OTA (qn)	6.4	404	239	40	10	33	16
ROQ (ql)	4.9	390.2	322.2	30	10	30	15
ROQ (qn)	4.9	390.2	193.1	30	10	38	15
STER (ql)	7.0	325.1	310.2	40	10	35	18
STER (qn)	7.0	325.1	281.1	40	10	51	16
T-2 (ql)	5.7	484.3	185.1	40	10	27	11
T-2 (qn)	5.7	484.3	215.2	40	10	25	18

qn: quantifier ions. ql: qualifier ions

Table S6
Instrumental MS/MS parameters of mycotoxins analysed in negative ionisation mode

Component	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
3AcDON (qn)	3.4	397.1	337	-20	-10	-12	-23
3AcDON (ql)	3.4	397.1	59	-20	-10	-52	-7
AOH (qn)	6.0	257	212.9	-95	-10	-34	-11
AOH (ql)	6.0	257	214.9	-95	-10	-36	-11
AME (qn)	7.2	271.1	256	-90	-10	-32	-13
AME(ql)	7.2	271.1	227	-90	-10	-50	-9
CIT (qn)	4.4	281.2	249.2	-50	-10	-30	-8
CIT (ql)	4.4	281.2	205.2	-50	-10	-37	-8
DON3G (qn)	2.7	517.2	457.1	-45	-10	-18	-13
DON3G (ql)	2.7	517.2	247	-45	-10	-32	-17
MON (qn)	0.9	96.6	41.2	-95	-10	-27	-5
NPA (qn)	1.0	118	46	-35	-10	-16	-15
NIV (qn)	2.5	371.1	281	-55	-10	-20	-19
NIV (ql)	2.5	371.1	311	-55	-10	-14	-21
PA (qn)	3.0	168.9	110	-40	-10	-12	-9
PA (ql)	3.0	168.9	92.9	-40	-10	-22	-9
β-ZEL (qn)	6.0	319.3	160	-110	-10	-41	-13
β-ZEL (ql)	6.0	319.3	130	-110	-10	-47	-20
ZEN (qn)	6.5	317.1	175	-175	-10	-32	-15
ZEN (ql)	6.5	317.1	131.1	-175	-10	-36	-11
α-ZEL (qn)	6.6	319.2	160	-110	-10	-41	-13
α-ZEL (ql)	6.6	319.2	130	-110	-10	-47	-20

qn: quantifier ions. ql: qualifier ions

Table S7
Recovery per mycotoxin (based on QC samples) in different samples

Compound	AFB_1	AFB_2	AFG_1	AFG_2	AFL	AFP_1	AFM_1	AFQ_1
Maize	87%	102%	92%	93%	85%	86%	83%	89%
Substrate	98%	88%	88%	88%	91%	93%	93%	96%
Larvae	97%	101%	93%	98%	88%	94%	99%	87%
Frass	104%	105%	118%	102%	95%	84%	98%	97%
Compound	3AcDON	15AcDON	AC	BEA	DON	DAS	ENNA	$ENNA_1$
Maize	71%	92%	85%	87%	77%	60%	106%	102%
Substrate	87%	96%	85%	88%	90%	92%	113%	116%
Larvae	101%	90%	83%	45%	84%	92%	48%	68%
Frass	98%	109%	100%	97%	112%	109%	120%	121%
Compound	ENNB	$ENNB_1$	HT-2	MPA	OTA	PA	ROQ	STER
Maize	102%	112%	124%	102%	95%	108%	84%	94%
Substrate	111%	121%	89%	89%	93%	105%	88%	89%
Larvae	64%	75%	95%	94%	90%	87%	74%	87%
Frass	137%	132%	107%	111%	102%	99%	101%	106%
Compound	T-2	AOH	AME	DON3G	NPA	NIV	ZEN	α-ZEL
Maize	60%	72%	81%	73%	82%	71%	77%	65%
Substrate	92%	92%	90%	88%	103%	96%	92%	82%
Larvae	92%	91%	82%	74%	69%	80%	88%	84%
Frass	112%	81%	113%	81%	104%	94%	90%	90%
Compound	β-ZEL	CIT	FB_1	FB_2	FB_3	MON		
Maize	69%	264%	99%	95%	97%	92%		
Substrate	101%	119%	98%	103%	101%	111%		
Larvae	98%	112%	117%	118%	114%	165%		
Frass	72%	91%	95%	92%	93%	102%		

Table S8
Limit of quantification (LOQ) and limit of detection (LOD) of all mycotoxins analysed in this study in µg/kg

Parameter	LOQ				LOD			
	Raw maize	Substrate	Larvae	Frass	Raw maize	Substrate	Larvae	Frass
AFB_1	0.29	0.25	0.26	0.24	0.14	0.13	0.13	0.12
AFB_2	0.12	0.14	0.12	0.12	0.12	0.14	0.12	0.12
AFG_1	0.14	0.14	0.13	0.11	0.09	0.09	0.08	0.07
AFG_2	0.54	0.57	0.51	0.49	0.13	0.14	0.13	0.12
AFL	1.55	1.45	1.5	1.38	0.46	0.44	0.45	0.41
AFM_1	1.06	0.95	0.89	0.9	0.32	0.28	0.27	0.27
AFP_1	1.02	0.94	0.94	1.04	0.46	0.42	0.42	0.47
AFQ_1	0.99	0.92	1.01	0.91	0.3	0.28	0.3	0.27
15AcDON	21.7	20.83	22.13	18.3	10.85	10.42	11.06	9.15
3AcDON	56.47	45.73	39.58	40.8	28.24	22.87	19.79	20.4
DAS	4.14	2.71	2.72	2.29	1.03	0.68	0.68	0.57
DON	32.3	27.79	29.82	22.41	32.3	27.79	29.82	22.41
DON3G	137.19	113.1	135.78	123.31	102.89	84.82	101.84	92.48
HT2	4.03	5.59	5.27	4.67	2.01	2.8	2.63	2.34
NIV	141.18	103.9	125.04	106.23	105.88	77.92	93.78	79.67
T2	4.15	2.72	2.72	2.23	4.15	2.72	2.72	2.23
α-ZEL	15.46	12.22	11.84	11.06	7.73	6.11	5.92	5.53
ZEN	8.13	6.78	7.12	6.96	8.13	6.78	7.12	6.96
β-ZEL	14.53	9.94	10.15	13.91	7.26	4.97	5.08	6.96
FB_1	12.64	12.7	10.65	13.12	12.64	12.7	10.65	13.12
FB_2	5.28	4.83	4.24	5.42	5.28	4.83	4.24	5.42
FB_3	5.18	4.93	4.38	5.39	5.18	4.93	4.38	5.39
ENNA	5.87	5.53	13.03	5.22	5.87	5.53	13.03	5.22
$ENNA_1$	6.13	5.4	9.21	5.15	6.13	5.4	9.21	5.15
ENNB	6.15	5.61	9.77	4.58	6.15	5.61	9.77	4.58
$ENNB_1$	5.58	5.16	8.35	4.72	5.58	5.16	8.35	4.72
BEA	2.87	2.83	5.52	2.56	1.44	1.41	2.76	1.28
MON	3.41	2.81	1.89	3.06	3.41	2.81	1.89	3.06
NPA	6.13	4.84	7.21	4.8	3.06	2.42	3.61	2.4
STER	0.13	0.14	0.14	0.12	0.13	0.14	0.14	0.12
CIT	0.47	1.05	1.12	1.38	0.47	1.05	1.12	1.38
MPA	12.23	13.97	13.3	11.23	6.12	6.99	6.65	5.61
OTA	0.53	0.54	0.55	0.49	0.26	0.27	0.28	0.25
PA	55.32	57.21	68.68	60.48	13.83	14.3	17.17	15.12
ROQ	0.15	0.14	0.17	0.12	0.15	0.14	0.17	0.12
AME	1.55	1.39	1.53	1.1	0.77	0.7	0.76	0.55
AOH	3.45	2.72	2.73	3.09	3.45	2.72	2.73	3.09
AC	0.15	0.15	0. 15	0.13	0.15	0.15	0.15	0.13

Abbreviations see “Sample preparation, extraction and analysis”

Table S9
Moisture content and gross nutrient composition of substrates with two different maize inclusion levels (% DM except for moisture content in %)

Maize inclusion	Moisture content	Crude protein	Crude fat	Ash	Crude fibre
50% maize	66.3 (4.0)	8.4 (0.7)	5.4 (0.3)	3.0 (0.1)	2.9 (0.8)
80% maize	68.8 (1.9)	8.8 (0.7)	4.9 (0.1)	1.8 (0.3)	3.0 (0.7)

Average and standard deviation in parentheses of pooled sample from the three maize mycotoxin contamination levels (CM: control maize; LM: low mycotoxin-contaminated maize, HM: high mycotoxin-contaminated maize)

Table S10
Concentrations of mycotoxins found in the different rearing substrates in µg/kg DM

	CM50	LM50	HM50	CM80	LM80	HM80
Aflatoxins[1]						
AFB_1	0.4 (0.5)^{a}	16.2 (1.7)^{a}	41.0 (12.4)^{a}	1.5 (0.2)^{a}	18.8 (3.8)^{a}	99.4 (47.5)^{b}
AFB_2	< LOQ^{a}	2.5 (0.2)^{b}	7.5 (1.1)^{c}	< LOQ^{a}	2.7 (0.1)^{b}	12.2 (1.4)^{d}
AFG_1	0.4 (0.3)^{a}	3.7 (1.2)^{b}	8.6 (0.4)^{c}	1.0 (0.1)^{a}	3.3 (0.7)^{b}	11.3 (1.6)^{d}
AFG_2	< LOQ^{a}	< LOQ^{a}	2.7 (0.6)^{a}	< LOQ^{a}	< LOQ^{a}	0.5 (1.1)^{b}
Fusarium mycotoxins[2]						
DON	115.9 (3.6)^{a}	131.1 (6.3)^{a}	142.1 (19)^{a}	127.6 (7.8)^{a}	130.7 (7.5)^{a}	135.2 (17.3)^{a}
ZEN	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}	15.9 (31.7)^{a}
FB_1	109.7 (11.1)^{a}	347.4 (73.3)^{b}	493.3 (50.3)^{c}	215.8 (13.5)^{a}	469.2 (49.2)^{b,c}	1034.6 (93.5)^{d}
FB_2	37.8 (8.9)^{a}	121.8 (34.1)^{a,b}	169.2 (31.4)^{b}	66.8 (5.9)^{a,b}	156.3 (30.7)^{b}	378.6 (104.6)^{c}
FB_3	12.7 (8.6)^{a}	45.6 (12.6)^{a,b}	65.7 (5.3)^{a,b}	29.1 (1.4)^{a,b}	69.9 (9.2)^{b}	208.5 (55.9)^{c}
ENNB	< LOQ^{a}	< LOQ^{a}	9.2 (18.4)^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}
$ENNB_1$	< LOQ^{a}	< LOQ^{a}	4.6 (9.3)^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}
BEA	< LOQ^{a}	3.0 (5.9)^{a,b}	13.5 (1.4)^{b}	< LOQ^{a}	3.7 (7.3)a,b	28.1 (11)c
MON	36.1 (4.4)^{a}	54.9 (8.4)^{a,b}	68.3 (5.9)^{b}	54.6 (3.7)^{a,b}	79.2 (12.9)^{b}	193.5 (23.1)^{c}
Aspergillus, Penicillium, and Alternaria mycotoxins[3]						
NPA	< LOQ^{a}	< LOQ^{a}	47.8 (4.6)^{b}	< LOQ^{a}	< LOQ^{a}	67.4 (13.9)^{c}
STER	< LOQ^{a}	0.2 (0.5)^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ^{a}
CIT	< LOQ^{a}	0.9 (1.9)^{a}	8 (1.9)^{a}	< LOQ^{a}	0.9 (1.9)^{a}	23.2 (30.8)^{a}
AME	9.9 (0.9)^{a}	12 (1)^{a}	8.4 (0.6)^{a}	4.4 (5.3)^{a}	< LOQ^{a}	13.9 (24.6)^{a}
AOH	12 (0.9)^{a}	13.9 (1.8)^{a}	10.7 (1.4)^{a}	2.2 (4.3)^{a}	< LOQ^{a}	15.2 (30.5)^{a}

All values are based on dry mass; Average and standard deviation in parentheses with n=4; Results with no shared letter are significantly different from each other (comparing all treatments with each other); [1]aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2); [2]deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), beauvericin (BEA) and moniliformin (MON); [3]nitropropionicacid (NPA), sterigmatocystin (STER), citrinin (CIT), alternariol-monomethylether (AME) and alternariol (AOH)

Table S11
Mycotoxin concentrations in substrate, larvae and frass with the clean maize in µg/kg DM

	CM50			CM80		
	Substrate	Larvae	Frass	Substrate	Larvae	Frass
AFB_1	0.4 (0.5)[a]	< LOQ[a]	0.2 (0.3)[a]	1.4 (0.2)[b]	< LOQ[a]	1.2 (0.2)[b]
AFG_1	0.4 (0.3)[b]	< LOQ[a]	< LOQ[a]	1.0 (0.1)[b]	0.2 (0.3)[a]	0.7 (0.1)[b]
DON	115.9 (3.6)[c]	< LOQ[a]	69.4 (15.1)[b]	127.6 (7.8)[c]	< LOQ[a]	112.6 (7.9)[b]
FB_1	109.7 (11.1)[b]	46.9 (9.1)[a]	125.4 (36.3)[b]	215.8 (13.5)[b]	41.6 (31.0)[a]	252.1 (15.1)[b]
FB_2	37.8 (8.9)[b]	15.5 (2.6)[a]	40.6 (11.8)[b]	66.8 (5.9)[b]	15.9 (11.5)[a]	76.7 (1.4)[b]
FB_3	12.7 (8.6)[b]	< LOQ[a]	21.3 (1.3)[b]	29.0 (1.4)[b]	< LOQ[a]	32.2 (1.0)[c]
MON	36.1 (4.4)[c]	< LOQ[a]	10.6 (1.1)[b]	54.6 (3.7)[b]	< LOQ[a]	< LOQ[a]
AME	9.9 (0.9)[b]	1.1 (2.1)[a]	14.9 (2.2)[c]	4.4 (5.3)[a]	< LOQ[a]	5.1 (1.0)[a]
AOH	12.0 (0.9)[b]	< LOQ[a]	20.9 (2.0)[b]	2.2 (4.3)[a]	< LOQ[a]	8.5 (1.5)[b]
BEA	< LOQ	< LOQ	< LOQ	< LOQ[a]	< LOQ[a]	5.0 (3.4)[b]

All values are based on dry mass; Average and standard deviation in parentheses with n=4; Results with no shared letter are significantly different from each other (comparing all treatments with each other); Results < LOQ were set to 0; Aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), moniliformin (MON), alternariol-monomethylether (AME), alternariol (AOH) and beauvericin (BEA)

Table S12
Mycotoxin concentrations in substrate, larvae and frass at the low maize mycotoxin contamination level in µg/kg DM.

	LM50			LM80		
	Substrate	Larvae	Frass	Substrate	Larvae	Frass
AFB_1	16.2 (1.7)^{b}	< LOQ^{a}	14.4 (7.8)^{b}	18.8 (3.8)^{b}	3.6 (1.2)^{a}	15.8 (1.0)^{b}
AFB_2	2.5 (0.2)^{b}	< LOQ^{a}	1.7 (0.7)^{b}	2.7 (0.1)^{b}	0.4 (0.3)^{a}	2.1 (0.2)^{b}
AFG_1	3.7 (1.2)^{b}	0.1 (0.2)^{a}	1.7 (1.2)^{a,b}	3.3 (0.7)^{b}	0.3 (0.4)^{a}	2.1 (0.2)^{a,b}
AFP_1	< LOQ^{a}	< LOQ^{a}	0.8 (1.7)^{a}	< LOQ^{a}	< LOQ^{a}	3.5 (1.0)^{a}
DON	131.1 (6.3)^{c}	< LOQ^{a}	88.9 (6.4)^{b}	130.7 (7.5)^{c}	< LOQ^{a}	126.6 (12.9)^{b}
FB_1	347.4 (73.3)^{b}	84.9 (13.4)^{a}	444.6 (17.4)^{c}	469.2 (49.2)^{b}	99.4 (30.6)^{a}	608 (96.3)^{c}
FB_2	121.8 (34.1)^{b}	28.6 (4.9)^{a}	151.0 (9.6)^{b}	156.3 (30.7)^{b}	37.6 (7.4)^{a}	188.7 (22.1)^{b}
FB_3	45.6 (12.6)^{b}	3.5 (7)^{a}	58.5 (3.5)^{b}	69.9 (9.2)^{b}	15.3 (3.5)^{a}	82.1 (14.3)^{b}
BEA	3 (5.9)^{a}	< LOQ^{a}	10.2 (1.7)^{b}	3.7 (7.3)^{a}	< LOQ^{a}	12.4 (1.3)^{b}
MON	54.9 (8.4)^{b}	< LOQ^{a}	< LOQ^{a}	79.2 (12.9)^{b}	< LOQ^{a}	3.7 (4.3)^{a}
STER	0.2 (0.5)^{a}	< LOQ^{a}	< LOQ^{a}	< LOQ	< LOQ	< LOQ
CIT	0.9 (1.9)^{a}	< LOQ^{a}	1.4 (2.7)^{a}	0.9 (1.9)^{a}	< LOQ^{a}	< LOQ^{a}
AME	12 (1)^{b}	< LOQ^{a}	16.5 (1.9)^{c}	< LOQ^{b}	< LOQ^{a}	4.8 (0.6)^{c}
AOH	13.9 (1.8)^{b}	< LOQ^{a}	24 (3.1)^{c}	< LOQ^{b}	< LOQ^{a}	6.3 (4.2)^{c}

All values are based on dry mass; Average and standard deviation in parentheses with n=4; Results with no shared letter are significantly different from each other (comparing all treatments with each other); Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin P_1 (AFP_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), beauvericin (BEA), moniliformin (MON), sterigmatocystin (STER), citrinin (CIT), alternariol-monomethylether (AME) and alternariol (AOH)

Table S13
Mycotoxin concentrations in substrate, larvae and frass at the high maize mycotoxin contamination level in µg/kg DM

	HM50			HM80		
	Substrate	Larvae	Frass	Substrate	Larvae	Frass
AFB_1	41 (12.4)^{b}	3.4 (3.2)^{a}	38.5 (12.8)^{b}	99.4 (47.5)^{c}	11.4 (11.8)^{a}	62.1 (18.8)^{b}
AFB_2	7.5 (1.1)^{b}	0.4 (0.6)^{a}	6.4 (0.5)^{b}	12.2 (1.4)^{b}	1.4 (1.5)^{a}	9.8 (1)^{b}
AFG_1	8.6 (0.4)^{c}	0.4 (0.7)^{a}	4.5 (0.7)^{b}	11.3 (1.6)^{c}	1.5 (1.4)^{a}	7.8 (0.8)^{b}
AFG_2	2.7 (0.6)^{b}	< LOQ^{a}	1.9 (0.5)^{b}	0.5 (1.1)^{a,b}	< LOQ^{a}	1.7 (0.4)^{b}
AFP_1	< LOQ^{a}	< LOQ^{a}	12.1 (2.8)^{b}	< LOQ^{a}	< LOQ^{a}	10.1 (1.1)^{b}
DON	142.1 (19)^{c}	< LOQ^{a}	108.5 (10.1)^{b}	135.2 (17.3)^{c}	< LOQ^{a}	105.1 (5.1)^{b}
FB_1	493.3 (50.3)^{a}	232.7 (99.7)^{a}	1067.8 (206.5)^{b}	1034.6 (93.5)^{b}	296 (85.2)^{a}	1064.6 (79.9)^{b}
FB_2	169.2 (31.4)^{b}	73.5 (30.8)^{a}	365.5 (69.3)^{c}	378.6 (104.6)^{b}	114.3 (31.1)^{a}	363.7 (25.2)^{b}
FB_3	65.7 (5.3)^{a}	30.2 (12.4)^{a}	132.2 (29.1)^{b}	208.5 (55.9)^{b}	54.6 (16.2)^{a}	191.5 (12.9)^{b}
ENNB	9.2 (18.4)^{b}	< LOQ^{a}	< LOQ^{a}	< LOQ	< LOQ	< LOQ
$ENNB_1$	4.6 (9.3)^{b}	< LOQ^{a}	< LOQ^{a}	< LOQ	< LOQ	< LOQ
BEA	13.5 (1.4)^{a}	20.9 (14.3)^{a}	26.2 (4.3)^{a}	28.1 (11)^{a}	44.2 (15.8)^{a}	31.1 (5.7)^{a}
MON	68.3 (5.9)^{b}	< LOQ^{a}	< LOQ^{a}	193.5 (23.1)^{b}	< LOQ^{a}	18.2 (1.9)^{a}
NPA	47.8 (4.6)^{c}	< LOQ^{a}	35.1 (8.6)^{b}	67.4 (13.9)^{b}	< LOQ^{a}	< LOQ^{a}
STER	< LOQ^{a}	< LOQ^{a}	0.5 (0.1)^{b}	< LOQ^{a}	< LOQ^{a}	0.3 (0)^{a}
CIT	8 (1.9)^{b}	< LOQ^{a}	10.9 (2.2)^{b}	23.2 (30.8)^{a}	< LOQ^{a}	10.2 (3.5)^{a}
AME	8.4 (0.6)^{b}	< LOQ^{a}	13.9 (2.7)^{c}	13.9 (24.6)^{a}	< LOQ^{a}	5.3 (0.8)^{a}
AOH	10.7 (1.4)^{b}	< LOQ^{a}	21.6 (1)^{c}	15.2 (30.5)^{a}	< LOQ^{a}	10 (1.3)^{a}
ZEN	< LOQ	< LOQ	< LOQ	15.9 (31.7)^{a}	< LOQ^{a}	< LOQ^{a}

All values are based on dry mass; Average and standard deviation in parentheses with n=4; Results with no shared letter are significantly different from each other (comparing all treatments with each other); Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin P_1 (AFP_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), beauvericin (BEA), moniliformin (MON), nitropropionicacid (NPA), sterigmatocystin (STER), citrinin (CIT), alternariol-monomethylether (AME), alternariol (AOH) and zearalenone (ZEN)

Table S14
BSFL gross nutrient composition in comparison to fishmeal and soybean meal sampled in Rwanda (% DM)

Feed type	Crude protein	Crude fat	Ash	Crude fibre
Larvae (50% DM maize)	26.1 (2.2)	23.4 (1.6)	3.8 (0.3)	15.7 (0.6)
Larvae (80% DM maize)	29.4 (4.1)	23.1 (1.9)	4.2 (0.4)	16.7 (0.6)
Fishmeal	42.0	15.8	20.6	5.6
Soybean meal	13.2	5.0	4.2	25.4

Average and standard deviation in parentheses of pooled samples (all replicates per maize inclusion level) from the three maize mycotoxin contamination levels (CM, LM, HM). Single biological replicate per substrate.

Table S15
Literature summary of mycotoxins in BSFL feeding trials (µg kg DM^{-1})

Reference	Substrate type	Contamination type	Mycotoxin concentrations		
			Substrate	Larva	Frass
This study	Maize and agri-food by-products	Natural	AFB_1: 16.2-99.4 AFB_2: 2.5-12.2 AFG_2: 0.5-2.7 DON: 130.7-142.1 FB_1: 347-1,035 FB_2: 122-379 ZEN: < 6.8-15.9 OTA: < 0.54	AFB_1: < 0.3-11.4 AFB_2: < 0.1-1.4 AFG_2: < 0.5 DON: < 29.8 ZEN: < 7.1 FB_1: 85-296 FB_2: 29-114 OTA: < 0.5	AFB_1: 14.4-62.1 AFB_2: 1.7-9.8 AFG_2: 1.7-7.8 DON: 88.9-126.6 FB_1: 89-1068 FB_2: 151-365 ZEN: < 6.7-24.0 OTA: < 0.5
Purschke *et al*. (2017)	Maize and maize by-products	Natural	AFB_1:13.3 AFB_2: 2.6 AFG_2: 7.0 DON: 697.7 ZEN: 130.4 OTA: 39.4	< LOD (not provided)	AFB_1:10.9 AFB_2: < 4 AFG_2: < 16 DON: 1135.7 ZEN: 103.7 OTA: < 20
Leni *et al*. (2019)	Corn distillation residue and corn gluten feed	Natural	DON: 779-1,207 ZEN: 173 FB_1: 573-727 FB_2: 294-441	DON: < 10 ZEN: < 10 FB_1: < 25 FB_2: < 25	DON: < LOD-1,473 ZEN: < LOD-334 FB_1: < LOD-951 FB_2: < LOD-344
Bosch *et al*. (2017)	Chicken feed	Artificial	AFB_1: 13-415	AFB_1: < 0.0001	AFB_1: 10-1,270
Camenzuli *et al*. (2018)	Chicken feed	Artificial	AFB_1: 18-430 DON: 4,100-100,000 ZEN: 400-9,400 OTA: 80-2,000	AFB_1: < 1 DON: < 80-257 ZEN: < 20-27.5 OTA: < 1-3.9	AFB_1: 5.5-353 DON:7.7-317 ZEN: 0.7-35.3 OTA:0.2-5.1
Heuel *et al*. (2023)	Food waste	Artificial	AFB_1: 842	AFB_1: 4.0	Not analysed

LOQ: limit of quantification, LOD: limit of detection

Table S16
Legal mycotoxin limits (µg/kg relative to a feeding stuff with a moisture content of 12%) in food and feed in the East Africa Community (EAC) and the European Union (EU)

	Feed				Food	
Type	Maize	Complete feed	Complete feed	Insects	Maize grains	Maize grains
Region	EU	EU	EAC	EAC	EU	EAC[4]
Total aflatoxins	-	-	50-100[3] 200[4]	50[7]	10[8]	10[9]
AFB_1	20[1]	20[1]	10[5] 20[6]	20[7]	5[8]	5[9]
DON	12,000[2]	5,000[2]	-	-	75[8]	-
ZEN	3,000[2]	100-500[2,3]	-	-	750[8]	-
OTA	250[2]	10[2]	-	-	3[8]	-
Fumonisins (FB_1+FB_2)	60,000[2]	5,000-20,000[2,3]	-	-	2,000[8]	2,000[9]

[1]Legal values (EC 2002a), [2]Guidance values (EC 2006b), [3]poulty and fish, pig starter feed (RSB 2019a; RSB 2019b; RSB 2019c), [4]grower feed for pigs, finishing and lactating sow feed (RSB 2019a), [5]poultry and pig starter feed, fish feed (RSB 2019a; RSB 2019b; RSB 2019c), [6]grower feed for pigs, finishing and lactating sow feed and grower feed for pigs, finishing and lactating sow feed (RSB 2019b), [7]RSB (2022), [8]EC (2006b), [9]RSB (2017)

Figure S1
Pictures of the final materials used for formulation of a BSFL rearing diet. Left: maize flour following grinding of whole maize grains. Right: mixture of agri-food by-products

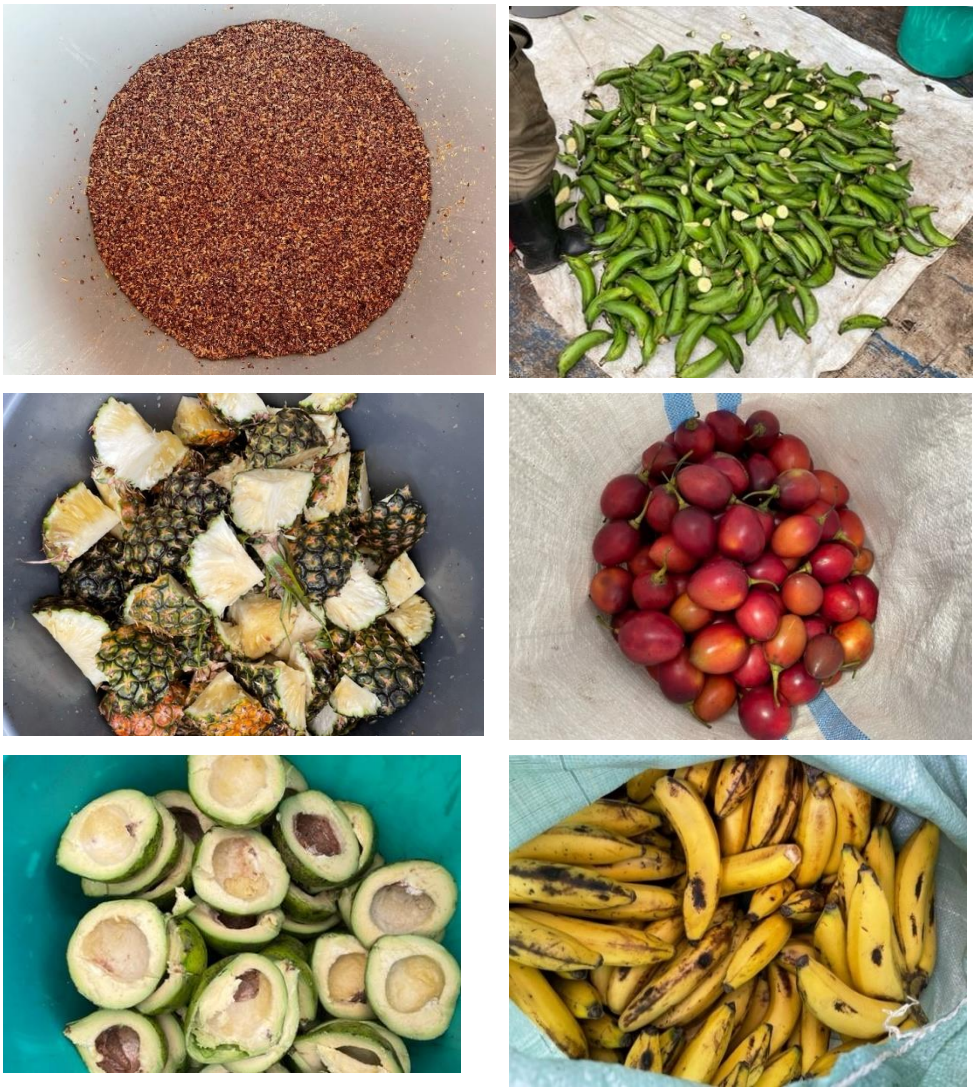

Figure S2
Pictures of raw materials making up the agri-food by-products used in this study. Top, left: spent grain. Top, right: green banana. Centre, left: pineapple. Centre, right: tree tomato, Bottom, left: avocado. Bottom, right: banana

Chapter 9

General discussion

In this thesis, we aimed to understand whether upcycling of mycotoxin-contaminated commodities, via insects into animal feed might contribute to global sustainability by ensuring food security and food safety. We proposed a circular approach (Figure 1) in which we aimed to feed larvae of the black soldier fly (BSF, *Hermetia illucens*) and housefly (HF, *Musca domestica*) with commodities contaminated with mycotoxins.

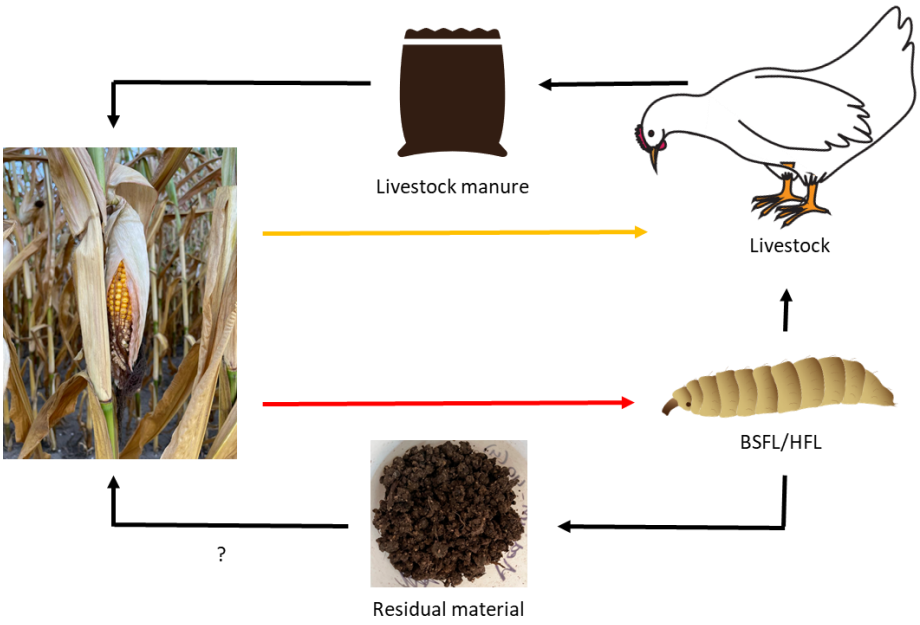

Figure 1. Proposed circular approach. The yellow line represents commodities contaminated with mycotoxins at concentrations higher than the maximum levels (MLs) or guidance values (GVs) for food, as defined by the European Commission, but lower than those for feed. The red line indicates the use of commodities that exceed the mycotoxin levels, *e.g.* MLs and GVs, for feed. The black lines show how insect larvae can be fed to animals such as pigs and poultry, and then their manure (frass) can be used to fertilize food crops. The material that remains from insect larvae that consumed mycotoxins could also be a fertilizer, but this is beyond the scope of this thesis. Pictures: own pictures.

First, in *Chapter 2* we performed a systematic literature review in which we summarized the scientific literature about mycotoxin exposure of insects, mycotoxin accumulation and biotransformation available until 2021 and aimed to identify knowledge gaps. Most data available were on insect species potentially used as food or feed in the EU and agricultural pests. We found that there were clear species-specific differences in the effects of mycotoxin exposure on survival, biomass, tolerance and metabolization. Tolerance differences were also observed between mycotoxins, their concentration, the type of substrate used, type of contamination (spiked or naturally contaminated), and the presence of supplements in the substrate. In general, literature showed that accumulation of mycotoxins in the insect body did not occur and was mostly below the limit of detection (LOD)/limit of quantification (LOQ) of the analytical method

used. Cytochrome P450s (CYP450s) were suggested as main enzymes involved in aflatoxin B_1 (AFB_1) metabolism in some insects; however, further research was recommended on unravelling metabolic pathways, involvement of phase II enzymes, the formation of possible unknown metabolites, and their toxicity. From the review we concluded that generalization of the already existing data is not possible and that further research on other insect species considered for food/feed, and mycotoxins of interest is necessary.

In *Chapter 2*, we identified two older studies on the effects of aflatoxin exposure on HFL, showing that accumulation of AFB_1 occurred in this species. This was contradicting the findings for other insect species. As HFL receive increasing attention as a possible feed source for livestock we therefore aimed in this thesis to reach the same level of understanding for HFL regarding the effects of mycotoxin exposure on their tolerance, accumulation and metabolism as was obtained previously for BSFL. We experimentally studied the effects of AFB_1, deoxynivalenol (DON) and zearalenone (ZEN) on survival, biomass and toxin accumulation in HFL in *Chapter 3*. In *Chapter 3*, we found that survival and biomass of HFL were not affected upon exposure to AFB_1, DON or ZEN in concentrations of 1x and 10x the ML or GV set for feed materials by the European Commission (EC 2002a; EC 2006b). Moreover, we found that accumulation in HFL did not occur for any of the mycotoxins in the concentrations tested. Metabolism of AFB_1 and ZEN did seem to occur as the molar mass balance revealed an unrecovered fraction of *ca.* 40 – 50%. Formation of the ZEN metabolites α- and β-zearalenol (ZEL) were observed in the residual material. On the other hand, most of the initially present DON was found back unchanged, indicating that little DON metabolism occurred. We concluded that further research is needed to identify the fate of the unrecovered fractions of AFB_1 and ZEN in the HFL-model.

In *Chapter 4* we studied the survival, biomass and mycotoxin accumulation of BSFL and HFL after exposure to either 0.1x or 1x the ML of regular AFB_1, fumonisin B_1 (FB_1), ochratoxin A (OTA), ZEN and their isotopically labelled versions ($^{13}C_{17}$-AFB_1, $^{13}C_{34}$-FB_1, $^{13}C_{20}$-OTA, $^{13}C_{18}$-ZEN). We anticipated to identify unknown novel metabolites via isotopic label-tracking in combination with HRMS analyses with the aim to obtain a full molar mass balance. Results reported in *Chapter 4* confirmed what was previously found regarding survival, biomass and mycotoxin accumulation for BSFL (Camenzuli *et al.* 2018) and HFL (*Chapter 3*) after feeding on substrates spiked with AFB_1, OTA (BSFL) or ZEN in a concentration of 1x the ML or GV. Also, similar results were found for feeding on a diet spiked with OTA (HFL) and FB_1 (BSFL and HFL) in concentrations of 1x its GV or 0.1x the GV, respectively. The recovered fraction of AFB_1 in the BSFL-model was higher (38%) than previously reported (4 - 17%; Bosch *et al*. 2017; Camenzuli *et al*. 2018), while for the HFL this was similar to what had been found in *Chapter 3*. In the BSFL study, formation of the AFB_1 metabolite aflatoxin P_1 (AFP_1) was observed and contributed (± 14 %) to the mass balance. Recovered fractions of 72% and 60% were found for FB_1 in the BSFL and HFL-models, respectively. The recovered fraction for FB in the BSFL was similar to what was found in another

study (Leni *et al.* 2019), while for the HFL this was the first time this species-toxin combination was studied. We identified that the metabolite ochratoxin-alpha (OTα) contributed for a large proportion to the recovery of OTA, and were, for the first time, able to fully reconstruct the molar mass balance for OTA for both the BSFL and the HFL. The mass balance of ZEN was complete for the BSFL, while a missing fraction of 42% remained for the HFL. α- and β-ZEL contributed to the mass balance in both insect species. Furthermore, isotopic label-tracking identified the formation of two possibly new zearalenone metabolites detected for both insect species. No novel metabolites were found for AFB_1, FB_1 or OTA. Overall, mass balances for AFB_1 (BSFL, HFL) and ZEN (HFL) remained incomplete. We hypothesized that it is possible that additional (unknown) metabolites *e.g.* larger or more polar molecules, as for example, metabolites conjugated with glucoside or sulphate were not extracted as effectively in the used extraction method. As we might have not extracted such metabolites with the currently used methods, they could, partly of fully, explain this missing fraction.

Afterwards, we wanted to examine whether enzymatic degradation of AFB_1 was caused by insect enzymes and/or by microbial enzymes of MOs in the feed substrate and whether non-enzymatic degradation took place. In *Chapter 5*, we observed that AFB_1 degradation can also take place in the substrate without the presence of larvae. However, AFB_1 degradation was more efficient in the treatment with BSFL as compared to the treatment without the larvae from day nine onwards, whereas no difference was observed for the HFL treatments. AFB_1 was differently metabolized in treatments with- (AFP_1) and without BSFL (aflatoxicol (AFL)), which indicated that both BSFL as well as substrate-specific MOs seem to play a role in AFB_1 metabolism. For HFL, the absence of aflatoxin metabolites in the residual material, and the fact that the percentual decrease of AFB_1 over time was not affected by the presence of HFL in the diets indicate that the HFL themselves do not have a role in AFB_1 degradation. Based on the collected data, it can be concluded that a longer exposure time results in a higher degradation and therefore a lower mass balance. No conclusion could be drawn on the occurrence of non-enzymatic degradation of AFB_1 in the feed substrates due to possible limitations in the extraction method used.

Throughout this thesis (*Chapter 4*, *Chapter 5*, *Chapter 7* and *Chapter 8*), formation of the AFB_1 metabolite AFP_1 has repeatedly been observed in BSFL studies. In *Chapter 5* we observed that formation of AFP_1 only occurred in the treatments with BSFL, which indicates that the BSFL, and not the substrate MOs, are responsible for this transformation. Formation of AFL only occurred in the treatments without BSFL, therefore, formation of AFL seems to be substrate-dependent. This is in contradiction with what was found by Meijer *et al*. (2019) who exposed S9 cell-free fractions of BSFL to AFB_1 and observed formation of both AFP_1 and AFL (Meijer *et al.* 2019). A recently conducted study confirmed that AFL was the main metabolite found after incubation of AFB_1 with a BSFL S9 fraction (personal communication, 2023). AFL can be reconverted back to AFB_1 and is therefore known to act as a reservoir for AFB_1 (Murcia and Diaz 2020b). It could

therefore be that conversion of AFB_1 into AFL by BSFL occurs during the first minutes/hours, and that AFL is converted back into AFB_1 or other metabolites before day seven, which is when we harvested the residual samples in our experiments. These findings highlight that results obtained *in vitro* cannot straightforwardly be extrapolated to *in vivo* conditions.

In general it can be stated that the substate is the main determinant of the larval gut bacterial community (Schreven *et al.* 2021). Over time, the larvae also alter the composition of the substrate bacterial community by inhibiting certain bacteria, changing the population sizes of resident bacteria, and by introducing gut bacteria into the substrate (Schreven *et al.* 2021). Furthermore, the impact of BSFL microbial composition of the substrate is substrate-dependent and bacterial communities in larvae and substrates can differ in composition depending on the feed substrate (Schreven *et al.* 2022). The use of the different feed substrates therefore result in different substrate- and insect-specific MOs and possibly a different AFB_1 degradation pattern. Formation of AFL was also observed in *Chapter 7*, therefore the substrate specificity of the MOs and their possibility to metabolize certain mycotoxins likely differs. Possibly, the conversion of AFB_1 into AFL occurs specifically in chickenfeed due to the microbiome that develops in it and/or the plant-derived enzymes it contains. Formation of AFM_1 was never observed in the residual material of BSFL feeding on AFB_1-spiked chickenfeed, and only occurred in peanut press cake (*Chapter 7*). However, in another study in which BSFL S9 extracts were used to test their potential to eliminate aflatoxins from naturally contaminated groundnut kernels, formation of AFM_1 was not observed (unpublished confidential report, 2018). On the other hand, formation of AFM_1 was observed in AFB_1 feeding experiments with the beetle *Alphitobius diaperinus* (Meijer *et al.* 2022). Whether *A. diaperinus* or the substrate MOs are responsible for this conversion cannot be concluded based on the existing data.

From literature it is known that CYP450 enzymes play an important role in mammalian AFB_1 metabolism (Bbosa 2013a). Their role in mycotoxin metabolism in a variety of insects has also been proven (Berenbaum *et al.* 2021; *Chapter 2*; Niermans *et al.* 2021). In *Chapter 5* we concluded that HFL do not contribute to AFB_1 metabolism and, therefore, we continued our search with BSFL in *Chapter 6*. In *Chapter 5* we could not distinguish between the role of the insect enzyme system and gut microbiota in AFB_1 metabolism. In *Chapter 6* we exposed BSFL to an AFB_1 spiked diet (1x ML) and collected larvae at three different timepoints (6 h, 48 h, 72 h) and extracted RNA which was sent for sequencing. RNA-Sequencing analyses showed that BSFL contain a wide variety of enzymes (*e.g.* CYP450 family and glutathione S-transferases) which are known to be involved in AFB_1 metabolism in mammals. Therefore, enzyme systems seem to be conserved amongst species. Furthermore, BSFL gene-expression data resulting from AFB_1 feeding gave a clear indication of three possible metabolic pathways *e.g.* CYP450 dependent demethylation into AFP_1, conjugation with glutathione (GSH) and glucose which seem to be involved in AFB_1 detoxification. We furthermore saw that there is a clear indication for a trade-

off of developmental *vs.* immunity-related gene expression in BSFL after feeding on an AFB_1-spiked diet, which could be detrimental when co-exposure with for example substrate pathogens occurs.

The first chapters of this thesis, as well as most of the studies published prior to this thesis, were performed with one or more spiked mycotoxins, on laboratory scale, with high quality substrates (*e.g.* poultry feed). In *Chapter 7* and *Chapter 8*, BSFL were fed on naturally contaminated substrates on both laboratory- and pilot scale. In *Chapter 7*, a feeding experiment was performed using peanut press cake (PPC) which was naturally contaminated with a mix of aflatoxins (AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1)), in concentrations below or around the respective MLs for animal feed (EC 2002a). Exposure to the aflatoxin-contaminated PPC did not affect survival and biomass of the BSFL, and for the first time, we found an average molar mass balance of 99 ± 26%, though a high variation between the replicates was noticed. Like the observations described in *Chapter 4*, formation of AFP_1, contributing with ± 11% of the molar mass balance, occurred in this study as well. Furthermore, as we aimed to understand whether upcycling of mycotoxin-contaminated local agricultural residual streams into protein-rich and low-mycotoxin insect biomass would be possible, we performed a feeding study in Kigali, Rwanda. In *Chapter 8* we showed that quality feed and fertilizer could be produced from naturally mycotoxin-contaminated crops mixed with agri-food by-products using BSFL in East Africa. We found that feeding on a substrate containing high concentrations of a mixture of various mycotoxins did not affect BSFL survival and biomass, but indicated that substrate composition *e.g.* maize inclusion level, seems to be most important for a good larval yield. For the first time we observed accumulation of the mycotoxin beauvericin (BEA) in BSFL, while accumulation of the other present mycotoxins did not take place. Additionally, we observed formation of AFP_1 in the residual material, confirming results from the BSFL studies performed in *Chapters 4* and *5*.

Insects in a circular system/economy

By now, various studies have shown that insects can help in upcycling 'waste' streams into high-quality animal protein (Gold *et al.* 2018; Lalander *et al.* 2019), while the residual material of the mass-rearing consisting of frass and exuviae can be used as a fertilizer for plants. Earlier studies suggested that insects are a more sustainable food and/or feed source as compared to conventional livestock, as they more efficiently convert feed to body mass (Nakagaki and Defoliart 1991; Ramos-Elorduy 2008). However, recent data showed that farmed insects are not always more efficient feed converters than livestock (Lalander *et al.* 2019; Parodi *et al.* 2020a; Parodi *et al.* 2021; Peters *et al.* 2014). Yield of larval biomass is directly affected by the suitability of the substrates, in particular their nutritional profile and other abiotic and biotic rearing conditions (Barragan-Fonseca *et al.* 2017; Lalander *et al.* 2019).

This we also observed in *Chapter 8*, where we observed that substrate composition *e.g.* maize inclusion level, seems to be most important for a high larval yield. Decreasing yields can affect profitability and process optimalisation and mixing of 'waste' substrates into more favourable nutritional profiles could be a solution. Insect value chains could only have environmental benefits if they replace compound feed production with very efficient systems (Smetana *et al.* 2023). For example, insects that are grown on left-over materials or low-cost feeds, and use alternative sources of heat and energy (Smetana *et al.* 2023). Moreover, insect farming will only support a sustainable circular economy if it uses feed that is not suitable for livestock. This includes manure (Parodi *et al.* 2022) and contaminated commodities that exceed the EU legal limits for contaminants *e.g.* mycotoxins. The EU currently doesn't allows this due to expected food- and feed safety issues.

Insect farming provides an alternative solution for mycotoxin-contaminated commodities by the opportunity to re-introduce part of these resources into the feed chain. Re-introduction of (mycotoxin) contaminated commodities and other wasted foods as substrates for insect farming into the cycle might prevent them from being either wasted or introduced in the informal food market, improves the sustainability of food systems, and contributes to food safety, and global food security. However, this will not be possible without changing regulatory frameworks in the EU. If the current regulations stay in force, insect production needs to compete with the conventional livestock for its feedstocks (Parodi *et al.* 2022). When enough experimental evidence on the safety of the proposed solutions is available, regulatory frameworks could be revised and adapted accordingly (Parodi *et al.* 2022). Moreover, insect farming will not reduce global mycotoxin contamination and food waste. Prevention of food waste should at all times be prioritised (Parodi *et al.* 2022).

Safety of insects as food and feed

This thesis and other work conducted in this research field show that in insects reared on mycotoxin- contaminated substrates mycotoxin accumulation generally (except for BEA, *Chapter 8*; Gold *et al*. 2023) not occurs (Bosch *et al.* 2017; Camenzuli *et al.* 2018; *Chapter 3*; *Chapter 4*; *Chapter 7*; Leni *et al.* 2019; Niermans *et al.* 2023a; Niermans *et al.* 2023b; Niermans *et al.* 2023c). This means that in general the larvae themselves are suitable as feed when considered under the EU legislation. However is needs to be kept in mind that when the larvae are fed on highly contaminated feed substrates some of the left-over feed in the larval gut might contain high mycotoxin concentrations. Nonetheless, it should be pointed out that levels of genotoxic carcinogens, such as AFB_1, in food and feed should generally be kept as low as possible.

In *Chapter 4* we observed that no novel unknown metabolites of AFB_1, FB_1, OTA or ZEN were present in the larvae, however, we also concluded that extraction methods could be optimized. We therefore advised to test the toxicity of extracts of BSFL and HFL fed on an mycotoxin-

contaminated diet *in vitro*. We have performed such a study in collaboration with the European SUSINCHAIN project. In the latter study (unpublished data) we have tested extracts of BSFL and HFL on an AFB_1-spiked diet (1x ML) for their cytotoxic and genotoxic potential in the Ames MPF™ assay. BSFL extracts were concluded not suitable for *in vitro* testing in this assay, due to matrix interferences, and no conclusion on the genotoxic potential of extracts of BSFL fed on an AFB_1 spiked diet could thus be made in that study. Extracts of the HFL showed no cytotoxic and genotoxic potential in the Ames MPF™ assay. The same HFL extracts were further evaluated by Charles River Laboratories Den Bosch BV (the Netherlands) which concluded that the HFL extracts did not induce mutagenicity (bacterial reverse mutation test; Ames test; OECD guidelines 471, non-GLP), and neither caused an increase in the number of micronuclei in human lymphoblastoid TK6 cells (Cryovial 300357, Vital: 330357, CLS, Eppelheim, Germany) which is a measure of DNA damage (*in vitro* micronucleus assay; non-GLP).

Similar results were found in a study in which *Tenebrio molitor* larvae were fed on an AFB_1-spiked diet in a much higher concentration (10,700 µg/kg). In this study the authors tested *T. molitor* extracts in another *in vitro* model (γH2Ax assay) in which histone H2Ax phosphorylation in human liver cells was analysed as an indicator for genotoxicity (Gützkow *et al.* 2021). The authors concluded that the *T. molitor* extracts did not exhibit an elevated toxic potential and that the resulting mutagenicity of the edible larvae appeared to be low (Gützkow *et al.* 2021). These two studies provide promising outlooks for the safe use of aflatoxin-fed insects as feed for livestock, however, generalizing between insects does not seem appropriate (*Chapter 2, Chapter 5*), and safety studies for *e.g*. BSFL are required.

Overall we conclude that HFL fed on an AFB_1-contaminated diet are safe to be used as feed for livestock due to the absence of accumulation and negative results for cytotoxicity, genotoxicity, mutagenicity and DNA damage when tested *in vitro*. However, we also learned that the development of proper methods for extracting compounds from insect biomass prior to *in vitro* bioassays is required. Furthermore, more research is needed to determine the cause of inhibition by insect matrices and how this can be prevented. Additional *in vitro* safety studies need to be performed on extracts from BSF and HF larvae fed on diets containing other mycotoxins and mycotoxin mixtures. *In vitro* assays need to be carefully selected and suitable for the expected mode of action of the respective mycotoxin. In *Chapter 4* we observed formation of the ZEN metabolites α- and β-ZEL in both insect species. The relative oestrogenic potency of α-ZEL is 60 times higher than for its parent compound ZEN, whereas β-ZEL has a relative potency of 0.2 as compared to ZEN (EFSA 2016). Even though ZEN and its metabolites did not accumulate in the larvae, subjecting such larval extracts to *in vitro* assays seems appropriate. Oestrogenic potential of the insect extracts can, for example, be investigated via an oestrogen receptor (ER) binding assay and reporter gene assays (Mukherjee *et al.* 2020), amongst others.

Insect welfare

During the past years, welfare in animal production systems including welfare of farmed insects, has received increasing attention (IPIFF 2019; Parodi *et al.* 2022; Van Huis 2019; Van Loon and Bovenkerk 2021). Increasing evidence led to infer that many insect species are sentient and possess cognitive and emotional abilities (Lambert *et al.* 2021; Parodi *et al.* 2020b). However, debates are ongoing on whether sentience *i.e.* being equated with phenomenal consciousness should be considered as a prerequisite for animal welfare or whether *e.g.* the frustration of desires (even though phenomenally unconscious) should be considered as a sufficient condition for moral standing (Van Loon and Bovenkerk 2021).

Currently, the European animal welfare Directive 98/58/EC for animal farming does not apply to insects and invertebrates (EC 1998). Ethicists argue that exclusion of farmed insects from such policies is not justified and that by the current state of evidence application of a precautionary principle (and therefore including farmed insects in the current legislation) would be suitable (Baracchi and Baciadonna 2020; RDA 2018; Van Loon and Bovenkerk 2021). This would mean that welfare standards for insect farming need to be developed and are required to guide good insect welfare in both commercial rearing systems and research (Van Loon and Bovenkerk 2021).

This thesis proposes the use of insects as a bioremediation or waste management tool to reduce food waste and improve food security by feeding them on a mycotoxin-contaminated diet, applications for which ethical considerations are relevant. Traditionally, biological markers *e.g.* health, growth and reproduction have been useful in monitoring animal welfare (RDA 2018). *Chapters 2, 3, 4, 5, 6* and *8* of this thesis provide evidence that survival and biomass of BSFL and HFL feeding on a mycotoxin-contaminated substrate are not affected. Thus far, only one study has observed a slower BSFL development with food waste containing a much higher (± 800 µg kg^{-1} DM) concentration of AFB_1 (Heuel *et al.* 2023).

However, welfare generally goes further than health, growth and reproduction, though such signs *e.g.* stress, pain, fear, natural behaviour, amongst others, are much more difficult to investigate (RDA 2018). Studies on insect behaviour in response to the presence of filamentous fungi or mycotoxins in the rearing substrate are existing but scarce. These studies showed that *Drosophila* adults did not avoid fungus-infested substrates as oviposition sites (Trienens *et al.* 2010), and that *Drosophila* larvae showed preference for fungal infested substrates rather than avoidance (Trienens *et al.* 2017). However, in *T. molitor* larvae fed on DON-spiked diets a dose-dependent decrease in locomotor activity (reduced time in movement and travel distance) was observed (Janković-Tomanić *et al.* 2019). Limited knowledge on 'normal' insect behaviours, especially regarding BSFL (Kortsmit *et al.* 2022), makes it difficult to determine whether such observations can be considered as indicators of stressed or abnormal behaviour.

From observations in *Chapter 6* we hypothesized that, although no larval mortality occurred in BSFL during the AFB_1 feeding trial, potential trade-offs due to increased investments in detoxification processes could result in developmental issues (low adult emergence, failure to emerge as adults or emergence of dysfunctional/malformed adults) and an impaired immunity. Impaired immunity can result in a lower resistance/increased susceptibility upon co-exposure with *e.g.* substrate-borne pathogens, which might affect BSFL survival and consequently could be detrimental in an insect rearing system. *Chapter 6* only focussed on exposure to AFB_1, and as we aim to grow larvae on naturally contaminated commodities, exposure to multiple mycotoxins may be expected to enhance detrimental effects. In general, chronic effects as shown in mammals, such as carcinogenicity, are not considered relevant in insects due to their much shorter life cycle (Gützkow *et al.* 2021). However, we believe that further studies in which pupation rate, adult emergence, oviposition and neonate hatching are studied after feeding on a mycotoxin-contaminated diet are warranted.

Mycotoxin contamination of agricultural commodities is of increasing concern. Based on a modelling study, the current probability of aflatoxin contamination was classified as very low to low in 93% of the grids representing Europe (Focker *et al.* 2023). For the current European situation, the aflatoxin problem and the ability of insect larvae to reduce it by about 22% (this may be an underestimate due to matrix interferences; *Chapter 5*) for both BSFL and HFL, seem not urgent enough to outbalance possible ethical issues. However, it is predicted that in the future (2050) aflatoxin contamination in maize cultivation in Europe will dramatically increase, particularly in Central and Southern Europe (Focker *et al.* 2023). This expected change in risk might allow use of insects for aflatoxin bioremediation as a suitable solution for the longer term, despite the possible welfare issues.

Furthermore, we believe that there are ample opportunities in the Global South where socio-economic issues caused by mycotoxin contamination are apparent and short-term solutions are needed. Insect rearing does not require big investments when executed on a community level. Currently, efficient techniques preventing mycotoxin contamination are non-existent, therefore using insects as a bioremediation tool to reduce food waste and improve food security and food safety by feeding them on a mycotoxin-contaminated diet may have a tremendous impact. We believe this application will limit the sales of mycotoxin-contaminated commodities on the informal market without wasting food sources, and positively affects global health due to a reduced consumption of mycotoxins. Furthermore, smaller scale (smallholder farmers at community level) insect rearing contributes to economic sustainability and social status, as proven in various initiatives (Barragán-Fonseca *et al.* 2020; Hanboonsong *et al.* 2013).

Ideally, current prevention and control measures for aflatoxin contamination are properly applied, and novel efficient techniques, such as the use of beneficial fungi as an active ingredient in biological control of mycotoxigenic fungi, are further developed in the near future. In order to

make our insect based solution, as well as such other initiatives successful, a more holistic approach is needed. Multidisciplinary teams including academia, the government and industry therefore need to work together to solve complex problems to improve health, society, and safeguard natural resources, amongst others (Garcia *et al.* 2020).

Methodological limitations

One of the main topics repeatedly discussed in this thesis is the importance of suitable extraction steps. For the mycotoxin analyses we used a QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction method. This method is an in-house method at Wageningen Food Safety Research (WFSR) which is validated according to SANTE/12682/2019 (EC 2019b). This method has been used as part of the statutory task of analyses of mycotoxins in animal feed samples at WFSR for years. However, the downside is that this method is optimized for the analyses of a specific set of mycotoxins [1]. This means that the used extraction method might not be optimal for extracting other mycotoxins or mycotoxin metabolites which are more polar or conjugated with *e.g.* sulphate, glucuronide or glutathione. Other studies have observed low aflatoxin recovery rates after spiking a food/feed matrix (Sulyok *et al.* 2020; Warth *et al.* 2012). This suggests that non-enzymatic conjugation of AFB_1 to, for example, the cell walls of substrate MOs, proteins in *e.g.* the plant matrix present in the feed substrates could be an additional cause of the incomplete mass balance. Resulting from this, extraction of mycotoxins in the residual material may have been less efficient with the currently used extraction method. Extraction efficiency and matrix effect of the substrate, larvae and residual material was repeatedly investigated throughout the experimental work reported in thesis, and was mostly acceptable or good. However, incubation time during an experiment varied between five to seven days whereas extraction efficiency was determined after incubation of maximally a couple of hours, and is therefore not fully representing the ''real'' situation. This unextracted fraction could account for a part of the missing fraction in the molar mass balance. Addition of a hydrolysis step or enzymatic treatment to free bound mycotoxins and/or conjugated metabolites during extraction, and/or analyses of the water phase obtained from the QuEChERS partitioning step might be essential. Optimization of the extraction method as well as the method of analyses for mycotoxin metabolites which are conjugated, but also DNA and/or protein adducts is therefore advised.

Feeding studies were conducted at a relatively small scale with three to six replicates per treatment in the current thesis, which made statistical validation challenging. To study multiple mycotoxins and insect species, a compromise was made by decreasing the number of replicates.

[1] 15AcDON, 3AcDON, AFB_1, AFB_2, AFG_1, AFG_2, agroclavine, alternariol, alternariol methylether, beauvericin, citrinin, DON, DON3G, diacetoxyscirpenol, enniatin A, enniatin A_1, enniatin B, enniatin B_1, fumonisin B_1, fumonisin B_2, fumonisin B_3, HT-2, moniliformin, mycophenolic acid, nitropropionic acid, nivalenol, ochratoxin A, penicillic acid, roquefortine C, β-ZEL, sterigmatocystin, T-2, ZEN, α-ZEL

In this thesis, we have mainly chosen to statistically analyse our data with non-parametric tests, as the criteria needed to perform parametric tests cannot be justified if the distribution type of the data cannot be verified due to the low number of replicates. Therefore the significance of possible interactions between factors could not always be investigated, however, we believe that the tests performed were informative for answering our research questions.

Implications for industry and government

Before using mycotoxin-contaminated substrates as feed for insect larvae can be considered as a safe and suitable solution, more research is required. Further studies on the hypothesized trade-offs due to increased investments in detoxification processes on insect development and immunity are needed. Such consequences could, but do not necessary have to be detrimental for insect-producing companies. However, the business model is a determining factor in this regard *e.g.* when the larvae are the end product further developmental effects may not be of interest. Additionally the safety of *e.g.* left-over mycotoxins present in the frass and its use as fertilizer (worker safety, leaching into the ground water, alternative exposure routes (via plant/agricultural commodity), further metabolism in the soil) needs to be investigated.

Furthermore, though both survival and biomass of the BSFL and HFL were not affected by mycotoxin exposure under the tested conditions, we did observe species (BSFL, HFL)- and substrate-specific differences regarding mycotoxin metabolism. HFL do not seem to play a role in the metabolism of AFB_1 (*Chapter 5*), but might be involved in the metabolism of other mycotoxins. Furthermore, the quick development time of HFL provides opportunities. It may however also provide difficulties; due to the quick development time using live HFL will not be an option in livestock feeding, while for BSFL this is possible. Furthermore, BSFL or HFL can be used to transform contaminated waste streams into valuable insect biomass and proteins, though it has been shown that the larvae play a minor role (± 22%) in aflatoxin bioremediation. Still, growing insects on these mycotoxin-contaminated streams, which are unsuitable for human and livestock consumption, results in valuable insect biomass. Investigation on whether transformation of mycotoxin- contaminated side-streams into *e.g.* biogas might be more sustainable or profitable is interesting, though this would not contribute to solving the food security issue. According to Moerman's ladder, which is a model that shows the 'optimum utilization' of residual flows, feeding animals with residual streams is usually more beneficial than converting them into biogas or other forms of energy (Waarts *et al.* 2011).

Results reported in *Chapter 6* of this thesis suggest BSFL possess the genetic repertoire to metabolize and degrade mycotoxins such as AFB_1. It could be interesting for the industry to explore the potential of these insect enzymes as a biological reagent. Bioinformatics could be used to investigate the possible suitability of enzymes of interest and their possibility to/efficiency to degrade certain mycotoxins.

Thus far it seems that HFL fed on an AFB_1 diet seem to be safe to feed to livestock, due to the absence of accumulation and negative results for *in vitro* cytotoxicity, genotoxicity, mutagenicity and DNA damage. We, however, do advise to perform additional *in vitro* toxicity assays on extracts of insects fed other mycotoxins, mycotoxin mixtures and naturally contaminated substrates. If the required future research proves that our proposed solution is safe, legislative changes need to be made in order to be able to implement this in Europe.

General conclusions

- The impact of mycotoxin exposure on survival and biomass differs between insect species.
- The metabolism of mycotoxins varies among insect species, therefore considering species-specific responses is important.
- Differences in tolerance between mycotoxins, their concentration, the type of substrate used, type of contamination (spiked or naturally contaminated), and the presence of supplements in the substrate were indicated.
- In our feeding studies with spiked substrates we observed that accumulation of the tested mycotoxins (aflatoxin B_1, fumonisin B_1, deoxynivalenol, ochratoxin A, zearalenone) did not occur in BSFL and HFL.
- When feeding BSFL a naturally contaminated diet containing various mycotoxins, only beauvericin accumulated.
- BSFL as well as substrate-specific microorganisms seem to play a role in aflatoxin B_1 metabolism, and the BSFL seem to be responsible for the partial metabolism of aflatoxin B_1 into aflatoxin P_1; on the contrary, HFL do not have a role in AFB_1 degradation.
- BSFL express a wide variety of enzymes (*e.g.* CYP450 family and glutathione S-transferases) required for aflatoxin B_1 metabolism.
- HFL fed on an aflatoxin B_1 spiked diet can be considered safe as livestock feed.

Recommendations and future outlook

During the past years the topic of insects as food and feed has received increasing attention and various aspects of the value chain have been studied. Questions have been answered, and new questions came up. Despite dedicating the past four years to comprehending the impact of feeding a substrate contaminated with mycotoxins on the survival and biomass of BSFL and HFL, as well as the occurrence of mycotoxin transfer, metabolism, and metabolic product formation, we have concluded that further research is necessary. This is particularly true with respect to method optimization and development.

The gene-expression data of BSFL from *Chapter 6* provided evidence of three potential metabolic pathways involved in the detoxification of AFB_1. Conducting feeding studies with AFB_1 and utilizing extraction and analytical methods that can extract and detect AFB_1 metabolites conjugated with glutathione (GSH), glucose, or AFB_1 mercapturate could be a relevant first step. Additionally, investigating the formation of DNA/protein adducts could be of interest. To obtain

a comprehensive understanding of AFB_1 degradation in insect feed substrates, tracking and identifying organic degradation substances such as carbon dioxide with ^{13}C isotopically labelled standards in respiratory experiments could provide more valuable insights. Additionally, *Chapter 6* showed that feeding on mycotoxin-contaminated substrates might affect insect welfare. However, the limited knowledge regarding 'normal' insect behaviours, particularly for BSFL, necessitates further studies to identify 'normal' behaviour before drawing conclusions on abnormal/stressed behaviours and insect welfare.

Further research is required to identify the cause of inhibition by insect matrices and to determine how it can be prevented. Additionally, feed safety studies should be conducted on extracts from BSF and HF larvae that have been fed diets containing other mycotoxins and mycotoxin mixtures.

In *Chapter 5* we were not able to distinguish between the role of the insect enzyme system and its gut microbiota in AFB_1 metabolism. Therefore studies on the role of the insect gut microbiota in mycotoxin degradation are needed. In order to do this, we can use antibiotics to inactivate specific bacteria present in the insect gut and/or inoculation of the feed substrate to increase the initial abundance of specific bacteria known to be associated with BSFL/HFL and/or their feed substrates to observe performance and/or formation of metabolites after this treatment (Gurung *et al.* 2019; Lin *et al.* 2015). Furthermore, gut bacteria reported to be associated with BSFL or HFL can be grown in mycotoxin-containing media to quantify bacterial multiplication in the presence of mycotoxins and the mycotoxin-metabolizing capacity of the bacteria with HPLC-MS/MS (De Almeida *et al.* 2017).

In the current thesis we only focussed on the upcycling of mycotoxin-contaminated substrates, however, organic streams *i.e.* manure and left-overs from restaurants or households, could contain a wide range of contaminants. However, also for these contaminants safety studies need to be conducted in order to guarantee the safety of the consumers later in the value chain. In the past years a lot regarding this topic has happened, and a variety of feeding studies have been performed. The effects of contaminants such as heavy metals and arsenic (Van der Fels-Klerx *et al.* 2016), veterinary drugs (Hoek-van den Hil *et al.* 2022), pharmaceuticals (Zhang *et al.* 2014), insecticides (Meijer *et al.* 2021a; Meijer *et al.* 2023b), plant toxins (Meijer *et al.* 2023a), per- and poly- fluoroalkyl substances (unpublished data SUSINCHAIN), packaging materials and microplastics (unpublished data SUSINCHAIN), but also the transfer of livestock DNA (unpublished data) have been investigated.

In the years to come, we anticipate gathering adequate data to determine whether the utilization of contaminated organic streams as substrates for insect rearing can be deemed safe. If it is deemed safe, we expect that legislative modifications will be implemented to enable its adoption in Europe.

Summary

Novel protein sources that are sustainable and circular are essential for animal feed production. Insect larvae, such as black soldier fly (BSFL; *Hermetia illucens* L.) and housefly (HFL; *Musca domestica* L.), have emerged as promising alternatives for animal feed during the past years. Insects offer several advantages, such as their ability to consume various organic residual streams and convert them into high-quality protein. However, such organic side-streams may be chemically contaminated by naturally occurring toxins *e.g.* mycotoxins. Mycotoxins are secondary fungal metabolites that frequently contaminate agricultural crops. Globally, large amounts of various crops such as cereals, oilseeds, nuts and spices are contaminated with mycotoxins during pre-harvest, postharvest handling, processing and/or storage. Mycotoxin exposure affects human and animal health and can cause both acute toxicity and chronic carcinogenicity. Border rejection due to mycotoxin contamination has financial consequences and directly impacts global trade. Furthermore, mycotoxin contaminations hamper global food safety and security, and valorisation options of contaminated crops are urgently needed.

In this thesis we proposed a circular approach in which we aimed to feed larvae of the BSF and HF with commodities contaminated with mycotoxins. However, the safety of insect biomass reared on mycotoxin-contaminated substrates should be evaluated before using it as feed or food. We, therefore, investigated whether survival and biomass of BSFL and HFL are affected after feeding on a mycotoxin-contaminated substrate and whether transfer of the mycotoxins from the rearing substrates into the larvae takes place. Furthermore, we studied whether insect larvae metabolise the mycotoxins fed and what metabolic products are formed.

Chapter 1 provides a general introduction on the need for novel and sustainable solutions to address the global challenges of food safety and food security. It presents various concepts and frameworks that have been proposed by the United Nations (UN) and the European Commission (EC) to tackle these issues, and especially focused on the Sustainable Development Goals (SDGs). In particular, it explains the concept of food security and food safety in relation to SDG2 (zero hunger). A brief overview on the food safety legislation in the European Union (EU) and the definition of different topics related to food contaminants, such as natural toxins, is given. This chapter furthermore explains that mycotoxins are globally main food contaminants, which cause serious health and economic problems. The main mycotoxins that are investigated in this thesis, such as aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxins and zearalenone (ZEN), as well as emerging mycotoxins and mycotoxin co-occurrence, are introduced. It also discusses the regulatory and guidance limits and the socio-economic impacts of mycotoxin contamination. Finally, the rationale for the proposal to use fly larvae as a potential solution for the global challenges of food safety and food security is presented. The chapter contains objectives and outlines of the thesis, offering an overview on the content of the next chapters.

Chapter 2 entails a systematic literature review that provides a comprehensive overview of the published evidence on effects of mycotoxin exposure on insect growth and survival, mycotoxin

accumulation within the insect body, and metabolization of various mycotoxins by insects. We furthermore provide an overview of enzyme systems (putatively) involved in mycotoxin metabolism in different insect species. The review includes 54 scientific articles published in the past 55 years, in total covering 32 insect species. We discuss the information per insect order *e.g.* Diptera, Coleoptera, Lepidoptera and ''other orders''. The main conclusions of the review are that insects of the order Coleoptera show lower mortality after exposure to aflatoxin B_1 (AFB_1) when compared to Lepidoptera and Diptera, and that effects of mycotoxins on larval growth and survival are less detrimental in later larval stages. Furthermore, this chapter shows that accumulation of mycotoxins is low in most insect species, and that mycotoxins are metabolized within the insect body, the degree of which depends on the particular mycotoxin and insect species. Cytochrome P450s (CYP450s) were identified as the main family of enzymes involved in biotransformation of mycotoxins in some insect species. The results obtained in this review provided the basis for the following experimental chapters.

Chapter 3 discusses a mycotoxin-feeding study performed with HFL on lab scale. In this study, the HFL were exposed to a feed substrate spiked with AFB_1, DON or ZEN at concentrations of either 1 or 10 times the maximum levels or guidance values allowed for feed materials as set by the EC. Mortality and biomass of HFL were recorded over five days of exposure. LC-MS/MS analysis was used to determine the concentration of the mycotoxins in the substrate offered, the larvae and the residual feed material. A molar mass balance was calculated to estimate how much of the spiked mycotoxins (and several metabolites) was recovered in the larval body and the residual material (mixture of faeces and unconsumed feed). Exposure to either of the three mycotoxins did not affect larval mortality and biomass, and accumulation in the larval body did not take place. Metabolism did seem to occur for AFB_1 and ZEN as the molar mass balance revealed an unrecovered fraction of *ca.* 40 – 50%. Little DON metabolism occurred as most of the initially present DON was found back unchanged.

Chapter 4 entails another lab-scale feeding study. As previous feeding experiments with spiked and naturally contaminated feed substrates indicated that formation of possible unknown metabolites of mycotoxins occurred in insects, tracking of conversion products through *e.g*. isotopic labelling was considered necessary. Therefore, in this chapter, BSFL and HFL were exposed to substrates spiked with regular and isotopically labelled AFB_1, fumonisin B_1 (FB_1), ochratoxin A (OTA) or ZEN. Exposure to the spiked substrates lasted either seven (BSF) or five (HF) days. Samples of the substrate, the residual material and the reared insects were analysed by LC-MS/MS and high resolution mass spectrometry (HRMS), in order to detect and quantify the mycotoxins and their metabolites present. The molar mass balance was found to be complete for OTA (BSFL, HFL) and ZEN (BSFL). For all tested mycotoxins insect survival and biomass were not affected and bio-accumulation in the insects did not occur. No isotopic labels were found back in unknown compounds except for two possibly new ZEN metabolites detected

in both insect species. Overall, mass balances for AFB_1 (HFL, BSFL) and ZEN (HFL) remained incomplete, indicating the need for further research.

In *Chapter 5* we investigated whether biotransformation of AFB_1 is caused by insect enzymes and/or by enzymes of microorganisms (MOs) in the feed substrate. Sterile- and non-sterile feed substrates were spiked with AFB_1 and incubated either with or without insect larvae (BSFL or HFL), and the AFB_1 concentration was recorded over time. Results showed that both BSFL and substrate-specific MOs play a role in the biotransformation of AFB_1 as well as in conversion of AFB_1 into AFP_1 and AFL, respectively. In contrast, HFL did not seem to contribute to AFB_1 degradation. We could not conclude whether non-enzymatic degradation of AFB_1 in the feed substrates occurred due to possible limitations in the extraction method.

In *Chapter 6* we aimed to identify the underlying mechanisms that allow BSFL to metabolize AFB_1. To elucidate these mechanisms, five-day-old BSFL were fed with a control or AFB_1-spiked diet. Larval samples were collected and subjected to RNA-Seq analysis to determine gene expression patterns. Provision of an AFB_1-spiked diet resulted in up-regulation of 357 and down-regulation of 929 unique genes. Upregulated genes included multiple genes that are known to be involved in AFB_1 metabolism in other (insect) species. Downregulated genes were generally involved in growth, development and immunity of the insects. Overall, BSFL possess a diverse genetic arsenal that encodes for enzymes capable of metabolizing AFB_1 without trade-offs for larval survival and growth. Negative effects of AFB_1 exposure on immunity-related processes are observed in the transcriptomic response indicative of a trade-off between detoxification and immune responses.

The first chapters of this thesis, as well as most of the studies published prior to this thesis, were performed with one or more spiked mycotoxins, on laboratory scale, with high quality substrates (*e.g.* poultry feed). Therefore in the last chapters we investigated whether our earlier findings could be extrapolated to a more realistic situation via the use of naturally contaminated substrates on both laboratory- and pilot scale.

In *Chapter 7* we explored whether naturally aflatoxin contaminated peanut press cake (PPC) would be a suitable rearing substrate for BSFL. Generally, groundnuts (peanuts; *Arachis hypogaea* L.) are used for vegetative oil production, which generate a variety of by-products, such as PPC, which consists of the solids remaining after oil pressing. BSFL were exposed to the PPC diets for eleven days. Data on larval survival and biomass were collected, and the PPC, BSFL and the residual material were analysed by LC-MS/MS to determine the concentration of eight different aflatoxins and known metabolites. Additionally, a bio-accumulation factor and a molar mass balance were calculated. BSFL survival and biomass were not affected by exposure to the aflatoxin-contaminated PPC diet. The aflatoxins did not accumulate in the insect body, which provides a promising outlook for the safety of rearing insects on aflatoxin contaminated PPC with

the purpose of using them as food- and/or feed. AFB_1 was the dominant compound found in PPC. Formation of aflatoxicol, aflatoxin P_1 (AFP_1), and aflatoxin M_1 occurred and taking these metabolites into account, a complete molar mass balance, thus full recovery, for AFB_1 was obtained. This result differs from previous studies using artificially spiked substrates in which no complete mass balance for AFB_1 could be constructed. This finding calls for additional studies comparing naturally contaminated with artificially spiked feedstuff to establish if the assumption that artificial spiking is representative for natural contamination is valid.

In *Chapter 8* we studied whether quality feed and fertilizer can be safely produced from naturally mycotoxin-contaminated crops using BSFL under realistic field conditions in East Africa. Naturally mycotoxin-contaminated maize (corn; *Zea mays* L.) was used as a model due its prevalence as food and feed and utilized by BSFL together with local agri-food by-products at a research facility in Rwanda. To assess the influence of the initial maize mycotoxin contamination and maize inclusion in the larval diet, feed substrates with three mycotoxin contamination levels and two maize inclusion levels were tested. BSFL were tolerant against the high mycotoxin concentrations (*e.g*. 99.4 µg aflatoxin B_1 per kg dry mass) as the presence of mycotoxins in the substrate did not affect BSFL biomass production. Product safety was assessed by quantifying the presence of 38 common and emerging mycotoxins and metabolites in the maize, substrates and BSFL products (*e.g*. larvae and residual material). The results show that it is possible to produce feed and fertilizer with BSFL that are considered safe within EU and East African legal limits from mycotoxin-contaminated maize commonly found in East Africa. For the first time we observed accumulation of the mycotoxin beauvericin in the BSFL.

In *Chapter 9*, we discuss the main results reported in the previous chapters and compare the outcomes of these studies with each other and other relevant literature. We observed clear trends across the chapters regarding the absence of effects on survival and biomass growth and the lack of bioaccumulation after feeding on a mycotoxin-contaminated diet in the insect larvae. Thus far, for only one of the mycotoxins studied (beauvericin) accumulation in the BSFL occurred. BSFL and their substrate-specific MOs contribute to the metabolism and degradation of AFB_1, while HFL do not show any evidence of AFB_1 metabolism. Furthermore, formation of the AFB_1 metabolite AFP_1 is common in BSFL studies. Additionally, it was observed that BSFL express a variety of enzymes involved in mycotoxin metabolism, such as CYP450s and glutathione S-transferases. This chapter also discusses the role of insects in a circular system and the concept of insect welfare, and some methodological limitations and challenges are addressed. The chapter concludes with implications for industry and government, recommendations for future research, and outlooks for the development of an insect-based circular economy.

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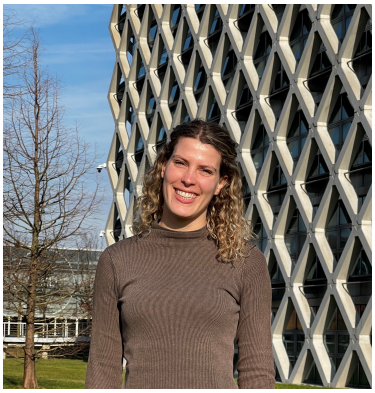
About the author

Curriculum Vitae



Kelly Niermans was born on July 16, 1993, in Amsterdam, The Netherlands. She completed her secondary education (VWO) at the Da Vinci College in Purmerend. In 2015, she graduated with a Bachelor of Nutrition and Dietetics from the Amsterdam University of Applied Sciences (HvA). During her studies, she completed two internships, one at the Research and Development department of the Archer Daniels Midland Company (ADM) in Zaandam, and the other at the Quality department of NewCakes BV in Wormerveer.

In 2015, Kelly began the MSc programme in Applied Food Safety at Wageningen University. She wrote her MSc thesis at Wageningen Food Safety Research (WFSR, formerly RIKILT) on the evaluation of the use of the Neuro-2a bioassay as an alternative method for the detection of diarrheic marine biotoxins. She also completed an internship at the National Reference Laboratory (NRL) for Mycotoxins in the Bundesinstitut für Risikobewertung (BfR) in Berlin, Germany, where she performed a feeding study investigating the effects of exposure to the mycotoxin zearalenone on yellow mealworm (*Tenebrio molitor*) larvae.

After successfully obtaining her MSc degree in 2018, Kelly worked as a scientist in the Department of Safety in the Food Chain at the BfR, where she continued the work she did during her internship. From December 2018 until November 2019, she did a traineeship in the BIOCONTAM unit at the European Food Safety Authority (EFSA) in Parma, Italy. Here, she supported the CONTAM working groups with the work on the scientific opinions regarding glycoalkaloids, chlorinated paraffins, meat inspection, hexabromocyclododecane, and polybrominated diphenyl ethers.

She began her PhD research in January 2020 at the Wageningen University Laboratory of Entomology and WFSR under the supervision of Prof. dr. ir. J. J. A. van Loon, Prof. dr. ir. H. J. van der Fels-Klerx, and dr. E. F. Hoek- van den Hil. Her research focused on studying the effects of mycotoxins in the feeding substrate on insect species reared for food and feed. During her PhD, Kelly pursued the postgraduate Education in Toxicology (PET) to become a European Registered Toxicologist (ERT). In March 2024, she began working as a researcher at the WFSR Agrochains group.

List of publications

Peer-reviewed publications

Niermans, K., Woyzichovski, J., Kröncke, N., Benning, R., Maul, R. (2019). Feeding study for the mycotoxin zearalenone in yellow mealworm (*Tenebrio molitor*) larvae - investigation of biological impact and metabolic conversion. Mycotoxin Research, 35(3):231-242. doi: https://doi.org/10.1007/s12550-019-00346-y

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Niermans, K., Hoek- van den Hil, E. F., Van Dam, R., Van der Fels-Klerx, H. J., Van Loon, J. J. A. (2023). Effects of the mycotoxins aflatoxin B_1, deoxynivalenol and zearalenone on survival, biomass and toxin accumulation in *Musca domestica* larvae. World Mycotoxin Journal doi: https://doi.org/10.1163/18750796-20222826

Gold, M., **Niermans, K.**, Jooste, F., Stanford, L., Uwamahoro, F., Wanja, M., Veldkamp, T., Sanderson, A., Dos Santos Nunes, V., Mathys, A., Van der Fels-Klerx, H. J., Hoek-van den Hil, E. F., Nishimwe, K. (2023). Conversion of mycotoxin-contaminated maize by black soldier fly larvae into feed and fertilizer. Journal of Insects as Food and Feed. doi: https://doi.org/10.1163/23524588-00001006

Submitted

Niermans, K., Meijer, N., Hoek- van den Hil, E. F., Nijssen, R., Zoet, L., Boers, E., Van Loon, J. J. A., Van der Fels-Klerx, H. J. (2024). The metabolic fate and biological effects of isotopically labelled aflatoxin B_1, fumonisin B_1, ochratoxin A and zearalenone in reared *Hermetia illucens* and *Musca domestica* larvae.

Niermans, K., Salari, S. P., Carney, J., Hoek- van den Hil, E. F., Van der Fels-Klerx, H. J., Van Loon, J. J. A. (2024). Conversion of aflatoxin-contaminated groundnut (*Arachis hypogaea* L.) press cake by larvae of black soldier fly *Hermetia illucens* results in a complete mass balance for aflatoxin B_1

Shah, P. N., **Niermans, K.**, Hoek- van den Hil, E. F., Dicke, M., Van Loon, J. J. A. (2024). Effects of aflatoxin B_1 on metabolism- and immunity-related gene expression in *Hermetia illucens* L. (Diptera: Stratiomyidae)

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (9 ECTS)

- Systematic review on the effect of mycotoxin exposure on the biological parameters, life-cycle and metabolism/conversion of mycotoxins by insects
- Insect health and substrate safety

Post-graduate courses (6 ECTS)

- PET Course: molecular toxicology; LACDR, Vrije Universiteit Amsterdam (2022)
- Insects as feed: interdisciplinary approach to insects as sustainable feed component; PE&RC Wageningen UR (2022)
- PET Training: organ toxicology; Radboud University Nijmegen (2023)
- PET Course: human exposure assessment; Radboud University Nijmegen (2023)

Laboratory training and working visits (4.5 ECTS)

- PET Training: pathobiology; UMC Utrecht (2020)
- PET Training: immunotoxicology; IRAS, Utrecht University (2021)
- PET Course: cell toxicology; Leiden University (2021)

Invited review of journal manuscripts (1 ECTS)

- JIFF: substrate safety/insects as food and feed (2021)

Deficiency, refresh, brush-up courses (10.5 ECTS)

- Insects as food and feed; Wageningen UR (2020)
- General toxicology; Wageningen UR (2020)
- Environmental toxicology; Wageningen UR (2023)

Competence, skills and career-oriented activities (2 ECTS)

- Project: testing the viability of aflatoxin-infected maize for black soldier fly consumption, Kigali, Rwanda skills in: communication and discussion/time planning and management/writing and presenting; Wageningen Food Safety Research, ETH Zürich, The University of Kigali, The Bug Picture, Rwanda Agriculture Board (2022)

Scientific integrity/ethics in science activity (2.1 ECTS)

- Scientific integrity; Wageningen UR (2020)
- PET Course: laboratory animal science; Utrecht University (2021)

PE&RC Annual meetings, seminars and the PE&RC weekend/retreat (1.5 ECTS)

- PE&RC First years weekend (2020)
- PE&RC Midterm weekend (2021)

Discussion groups/local seminars or scientific meetings (6 ECTS)

- PhD lunch meetings (2020-2024)
- Insect plant interactions entomology lunch meetings (2020-2024)
- WEES Seminars (2020-2024)
- Annual insectfeed meetings (2020-2024)

International symposia, workshops and conferences (6.3 ECTS)

- Insects to feed the world (2020)
- EAAP (2021)
- World mycotoxin forum (2022)
- EAAP (2023)

Societally relevant exposure (0.9 ECTS)

- Parma summerschool; online (2021)

Lecturing/supervision of practicals/tutorials (3 ECTS)

- Insects as food and feed (2021-2023)
- Fundamentals of plant pathology & entomology (2021-2023)

BSc/MSc thesis supervision (3 ECTS)

- Identification of the role of reared *Hermetia illucens*, *Musca domestica* and the rearing substrate on degradation and metabolism of aflatoxin B_1 in a spiked diet

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