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Effect of mycotoxin, deoxynivalenol, in aquaculture reared rainbow trout (*Oncorhynchus mykiss*) metabolism

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By

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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ABSTRACT

The decreasing supply and high cost of fishmeal, as well as the sustainability issues of this finite raw material, will continue to push the industry to concentrate their efforts on finding alternative sources of protein to substitute fishmeal in aquafeeds. From all the possible alternatives, e.g. animal by-products, fishery by-products, single-cell protein and the recent insect meal options, plant-based meals seem to be one of the major contributors to reduce reliance upon marine sources. However, when considering plant-based meals for aquafeeds it is commonly agreed that one of the negative aspects is the presence of anti-nutritional factors (ANF's; e.g. cyanogens, saponins, tannins, etc.) that are detrimental to fish and shrimp (Krogdahl *et al*., 2010). Although there are processes to remove or inactivate many of these ANF's, the same does not apply for mycotoxins which are reasonably stable to processing conditions (Cheli *et al*., 2013). The effects of mycotoxins in fish and shrimp are diverse, varying from immunosuppression to death, depending on toxin-related (type of mycotoxin consumed, level and duration of intake), animal-related (animal species, sex, age, general health, immune status, nutritional standing) and environmental-related (farm management, biosecurity, hygiene, temperature) factors. Therefore, it is often difficult to trace observed problems back to mycotoxins. The main goal of this thesis is to increase the awareness of mycotoxin contamination in aquafeeds and its consequences to aquaculture species, especially characterizing the impact of deoxynivalenol in rainbow trout (*Onchorhynchus mykiss*).

In **Chapter 1** the known mycotoxin occurrence and co-occurrence in aquaculture finished feeds are described and critically reviewed by correlating the extent of mycotoxin contamination in aquaculture feeds to its impact in aquaculture species. This chapter also contributes to understanding the risk of mycotoxin carry-over to aquaculture seafood products. Additionally, this chapter aims to expose the scientific community, the regulatory authorities and the aquaculture industry, to the main challenges and myths that the industry faces in developing mycotoxin management strategies.

Chapter 2 presents the results the impact of deoxynivalenol (DON) in rainbow trout and the difficulties to diagnose DON ingestion. Here was explored two different DON contamination scenarios, i.e., the effect of short-term feeding of high levels of DON and the effects of long-term feeding of low levels of DON (a more common scenario in

aquaculture industry). It was concluded that the ingestion of DON by trout over short-term periods at high dosages (50 days; 1,166 µg kg $^{-1}$ and 2,745 µg kg $^{-1}$) impacts growth performance, especially feed intake, with minor or variable biochemical changes in trout blood and histopathological changes. In this case, some fish did exhibit clinical symptoms (i.e., anal papilla), which could be attributed to the DON treatment (reported for the very first time). This chapter also reports, for the first time, the effects of the long-term exposure of rainbow trout to low concentrations of DON (168 days; 367 µg kg $^{-1}$ DON). Ingestion of DON in the long-term study was asymptomatic; however, the fish started to reduce their growth performance 92 days after ingesting DON. Such low contamination levels, which might be unnoticed by farmers, may have economic consequences for aquaculture. Here it was concluded that the short-term effect of DON ingestion cannot be extrapolated to other contamination scenarios, e.g., long-term exposure.

Chapter 3 aimed to elucidate the impact of DON on rainbow trout and study the reasons behind the apparent lack and/or high variability of clinical signs during DON ingestion (as reported in chapter 2). With this experiment, we further confirmed that ingestion of DON by rainbow trout is mainly characterised by impaired growth performance and reduced feed intake, with the total absence of any visible clinical signs (up to 11,412 ± 1,141 µg kg $^{-1}$). Proteolytic enzyme activities (pepsin, trypsin and chymotrypsin) in trout were altered by DON ingestion. The impact of DON on the abundance of specific measured mRNA transcripts was unexpected with higher expression levels for insulin-like growth factors, *igf1* and *igf2*, which are directly related to elevated insulin levels in plasma. This can also in part be influenced by the trypsin activity and by *npy*, given its higher mRNA expression levels. The apparent digestibility of dry matter, protein and energy was not affected by dietary levels of DON, however, nutrient retention, protein, fat and energy retention were significantly affected in animals fed DON. Adenylate cyclase-activating polypeptide (PACAP) expression seems to play an important role in controlling feed intake in DON fed trout. In the present study, we have shown for the first time that DON is metabolized to DON-3-sulfate in trout. DON-3-sulfate is much less toxic than DON, which helps to explain the lack of clinical signs in fish fed DON. Being a novel metabolite identified in trout makes it a potential biomarker of DON exposure. It was suggested that the suppression of appetite due to DON contamination in feeds might be a defence mechanism in order

to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON.

In **Chapter 4,** this thesis further explored the current knowledge of DON toxicokinetics and rainbow trout DON metabolism, accessing the organ assimilation rates, excretion and possible biotransformation kinetics. Here we found that an hour after tube-feeding, [^{3}H]-DON was detected in the liver samples of fish, indicating a rapid absorption of DON. In the first hour, [^{3}H]-DON was present in the GIT (20.56 ± 8.30 ng). However, 6.19 ± 0.83 ng was also detected in the water at this sampling point. The fast excretion of [^{3}H]-DON (faster than the average trout gastric emptying time) suggests that DON, as a water-soluble compound, is excreted with the liquid phase of the chyme. The presence of [^{3}H]-DON in the GIT was stable during the first six hours. This long residence time of DON in the GIT may compromise the health of the GIT and favour absorption. Our data suggest that an effective DON detoxifying method should have a period of action of ≤ 6 h. Furthermore, as most of the excretion can be expected to happen after six hours, the detoxification should be irreversible at GIT conditions.

Results of this PhD study contributes to better understand the importance and the risk of mycotoxin occurrence and co-occurrence in aquaculture finished feeds, highlighting concerns regarding the unvalued risk of mycotoxin carry-over to aquaculture seafood products. Here is highlighted that long-term exposure to DON is normally asymptomatic which might be unnoticed by farmers, however, representing economic consequences for aquaculture (reduced growth performance). Suppression of appetite due to DON contamination in feeds seems to be a defence mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The biotransformation of DON to DON-3-sulfate helps to explain the lack of clinical signs in fish fed DON and may be used as a novel biomarker of DON exposure.

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LIST OF ABBREVIATIONS

Mycotoxins abbreviations:

AFs: aflatoxins; meaning the sum of AFB_1, AFB_2, AFG_1 and AFG_2

AFB_1: aflatoxin B_1

AFB_2: aflatoxin B_2

AFG_1: aflatoxin G_1

AFG_2: aflatoxin G_2

DON: deoxynivalenol

ENNs: enniatins

FUM: fumonisins; meaning the sum of FB_1 and FB_2

FB_1: fumonisin B_1

FB_2: fumonisin B_2

OTA: ochratoxin A

ZEN: zearalenone

α-ZEL: alpha-Zearalenol

β-ZEL: beta-Zearalenol

DOM-1: deepoxy-deoxynivalenol

DON-3-sulfate: deoxynivalenol-3-sulfate

DOM-3-sulfate: deepoxy-deoxynivalenol-3-sulfate

Biological performance-related abbreviations:

ADC - apparent digestibility coefficients

GIT - gastrointestinal tract

ABW - average body weight

Other abbreviations:

Gene abbreviations:

ef1a - elongation factor 1 alpha

actb - beta actin

star - steroidogenic acute regulatory protein

Igf1 - insulin-like growth factor 1

Igf2 - insulin-like growth factor 2

crf1 - corticotropin releasing factor precursor 1

crf2 - corticotropin releasing factor precursor 2

crfbp - corticotropin releasing factor binding protein precursor

npy - neuropeptide Y precursor

adcyap1a - growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide (PACAP)

lep - leptin

sst2 - somatostatin-2 precursor

chia - gastric chitinase

pga - pepsinogen

lpl - lipoprotein lipase

ghrl - ghrelin/obestatin preprohormone

cel1 - carboxyl ester lipase 1

cel2 - carboxyl ester lipase 2

cckt - cholecystokinin (Tyrosine)

cckn - cholecystokinin (Asparagine)

cckl - cholecystokinin (Leucine)

amy2a1 - pancreatic alpha amylase

atp4a - ATPase H^+/K^+ transporting alpha subunit

crtl - chymotrypsinogen-like precursor

try1 - trypsinogen 1 precursor

try2 - trypsinogen 2 precursor

try3 - trypsinogen 3 precursor

Note: ZFIN Zebrafish Nomenclature Guidelines have been followed for all fish genes and proteins described in this thesis (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines#ZFINZebrafishNomenclatureGuidelines-1).

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CHAPTER 1

General introduction

1.1 Aquaculture production and its challenges

Aquaculture is the fastest-growing food production sector. The average growth of the sector was 5.8 per cent during the period of 2000 to 2016 (FAO, 2018). There is a clear maturation of the sector, although double-digit growth still occurs in a small number of individual countries, particularly in Africa. Global aquaculture production in 2016 reached 110.2 million tonnes, with a value estimated at USD 243.5 billion (including aquatic plants). From these, 80.0 million tonnes were of food fish (USD 231.6 billion; 54.1 million tonnes of finfish, 17.1 million tonnes of molluscs, 7.9 million tonnes of crustaceans and 938 500 tonnes of other aquatic animals), 30.1 million tonnes of aquatic plants (USD 11.7 billion)) and 37 900 tonnes of non-food products (USD 214.6 million) (FAO, 2018).

By comparison, global total capture fisheries production have been decreasing over the last two years, being in 2016 estimated to be 90.9 million tonnes (FAO, 2018). With most fishery stocks overfished or maximally sustainably fished, fisheries are not expected to increase, in the best case scenario it might be stabilized in current capture production. Therefore, aquaculture might be the solution to fill the growing gap between supplies of aquatic food and demand from a growing and wealthier global population. However, the growth of aquaculture raises a number of challenges in relation to the resources that it consumes (e.g. competition for space, feedstuffs namely fishmeal and fish oil), its product quality and security (e.g. food security, use of antibiotic growth promoters used), its sustainable growth (e.g. environmental footprint) and the threats that the sector faces from external factors such as climate change and diseases.

1.1.2 Fish meal: the aquaculture dilemma

Fishmeal and fish-oil production tend to fluctuate according to target species (e.g., anchoveta, *Engraulis ringens*) catch instabilities, which is highly affected by environmental phenomenon’s, such as El Niño, for example. Since the fishmeal production peak in 1994 (30 million tonnes live weight equivalent), an overall declining trend has been observed since then (FAO, 2018). In 2016, fishmeal production (from fisheries) were down to less than 15 million tonnes (live weight equivalent), mainly due to reduced catches of anchoveta (FAO, 2018). Better fisheries stock management has slowed the declining trend in fishmeal production but is unlikely to increase it in future. Following the market demand for fishmeal and oil, particularly for the aquaculture

industry, high prices have been observed for this commodity. This demand for fishmeal has pushed the industry to increase fishmeal used from fish by-products, which previously were often wasted. FAO (2018) estimates that fishe by-products accounted for about 25 to 35 per cent of the total volume of fishmeal and fish oil produced. However, regional variations are observable, for example, fish by-product use in Europe is comparatively high at 54 per cent (Jackson and Newton, 2016).

The use of fishmeal and fish oil in aquafeeds is more prevalent among higher trophic level species and crustaceans (Table S1.1). Fishmeal and fish oil use in low trophic level finfish species (e.g. carp, tilapia, catfish, milkfish) is also observable, however, at relative low inclusion levels (2 to 4 percent; Table S1.2). In general, it is observable that fishmeal replacement in lower trophic level species is relatively easier, compared to higher trophic levels species. Actually, it is already possible to observe commercial feed formulations with zero fishmeal (e.g. catfish, tilapia, milkfish), especially for grow-out stages. In 2015, the largest consumers of fishmeal were marine shrimp, followed by marine fish, salmon, freshwater crustaceans, fed carp, tilapia, eel, trout, catfish and miscellaneous freshwater fish and milkfish (Tacon *et al.*, 2011). The decreasing supply and high cost of fishmeal will continue to push the industry to concentrate their efforts on finding alternative sources of protein to substitute fishmeal in aquafeeds. From all the possible alternatives, e.g. animal by-products, fishery by-products, single-cell protein and the recent insect meal, plant-based meals seem to be one of the major contributors to reduce reliance upon marine sources.

1.1.3 The use of plant meals in aquafeeds

The growth of the worldwide aquaculture industry has been accompanied by the rapid growth of aquafeed production. In 2003, FAO estimated global aquafeed production of approximately 19.5 million tonnes but anticipating an increase to over 37.0 million tonnes by the end of that decade (FAO, 2004; Reus, 2017). In 2017, it is thought that feed produced for aquaculture could reach 39.9 million tons (Reus, 2017). Economic and sustainability factors have been the main forces pushing the aquaculture sector to identify economically viable and environmentally friendly alternatives to marine-derived ingredients, such as fish meal and fish oil. The inclusion of plant-based meals has been successfully achieved in several species with only minor negative consequences reported (Gatlin *et al.*, 2007). According to Tacon *et al.*, (2011), plant nutrients represent the major

dietary protein source used within feeds for lower trophic level fish species like tilapia, carp or catfish, and the second major source of dietary protein and lipids after fishmeal and fish oil for shrimp and European high trophic level fish species (Table S1.1 and S1.2). For example, tilapia feeds normally include soybean meal (20–60%), corn gluten meal (5–10%), rapeseed/canola meal (20–40 %), cottonseed meal (1–25%) and soybean oil (1–8%). Marine shrimp diets, normally have an inclusion of soybean meal (5–40%), wheat gluten meal (2–10%), corn gluten meal (2–4%), rapeseed/canola meal (3–20%) and lupin kernel meal (5–15%). In general, marine fish feeds include soybean meal (10–25%), soybean oil (3–6%), wheat gluten meal (2–13%), corn gluten meal (4–18%), sunflower seed meal (5–8%), rapeseed/canola meal (7–20%) and canola protein concentrate (10–15%). In trout and salmon, we can observe the use of more variable plant sources but include lower inclusion levels than in previous species. For example, trout feed commonly includes soybean meal (3–35%), wheat gluten meal (2–10 %), sunflower seed meal (5–9%), corn gluten meal (3–40%), rapeseed/canola meal (2–10%), lupin kernel meal (5–15%), fava bean meal (8%), field pea meal (3–10%), rapeseed/canola oil (5–15%) and soybean oil (5–10%). While salmon diets frequently include soybean meal (3–12%); wheat gluten meal (2–10%); sunflower seed meal (5–9%); corn gluten meal (10–40%); rapeseed/canola meal (3–10%); lupin kernel meal (5–15%); fava bean meal (5%); field pea meal (3%); rapeseed/canola oil (5–15%); soybean oil (5–10%) (Tacon *et al.*, 2011). However, when considering plant-based meals for aquafeeds it is commonly agreed that one of the negative aspects is the presence of anti-nutritional factors (ANF's; e.g. cyanogens, saponins, tannins, etc.) that are detrimental to fish and shrimp (Krogdahl *et al.*, 2010). Although there are processes to remove or inactivate many of these ANF's, the same does not apply for mycotoxins which are reasonably stable to processing conditions (Cheli *et al.*, 2013). The awareness of mycotoxin-related issues in the aquaculture industry has been increasing, accentuated by the increased inclusion levels of plant meals in aquafeeds (Gonçalves *et al.*, 2018d; Tacon *et al.*, 2011). Traditionally, the use of minor amounts of plant feed stuffs led to a general perception that mycotoxins were not a relevant issue in aquaculture and that the majority of mycotoxin issues would result from only due to poor storage conditions, i.e., aflatoxin contamination, which is not entirely correct (topic further explored in Chapter 1.2).

1.2 What are mycotoxins?

Mycotoxins are secondary metabolites produced by some moulds (Hussein and Brasel, 2001). These can be produced on agricultural commodities pre- and/or post-harvest including directly in finished feeds. Chemically, mycotoxins have low molecular weight and low immunogenic capacity (Mallmann and Dilkin, 2007), displaying a wide range of structures. These wide ranges of chemical structures are also responsible for its broad biological effects (e.g. carcinogenic - aflatoxin B_1, ochratoxin A, fumonisin B_1; estrogenic – zearalenone; neurotoxic - fumonisin B_1; nephrotoxic – ochratoxin; dermatotoxic - trichothecenes or immunosuppressive - aflatoxin B_1, ochratoxin A and T-2 toxin (Abd-Allah *et al.*, 1999; El-Sayed and Khalil, 2009; Hooft *et al.*, 2011; McKean *et al.*, 2006a). Despite being identified as categorically undesirable for most aquaculture species, their occurrence, at least in field conditions, is not completely preventable even when using good manufacturing practices.

Mycotoxins can cause diseases problems when consumed by humans and animals, causing significant problems worldwide (Zain, 2011). Some mycotoxicoses, the toxic manifestations of mycotoxins in humans or animals, have been known for hundreds of years, e.g. ergotism. It is thought that mycotoxins have plagued mankind since the beginning of controlled crop production if not earlier (FAO, 2001). They account for millions of American dollars lost annually worldwide due to negative impacts on human health, animal health, and condemned agricultural products (CAST, 2003; Hussein and Brasel, 2001; Shane and Eaton, 1994; Vasanthi and Bhat, 1998). Mycotoxins are chemically and thermally stable, rendering them unsusceptible to commonly used feed manufacturing techniques such as extrusion (Kabak *et al.*, 2006; Leung *et al.*, 2006). The most studied mycotoxins, economically affecting animal production, including aquaculture, are aflatoxins (AF's), deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA) and fumonisins (FUM). The focus of this thesis will be DON, which is a heat resistant compound with a melting point of 151 – 153 °C. Thermal processing does not lead to a significant reduction of DON levels in plant meals or finished feeds (CAC, 2003).

1.2.1 Mycotoxin-producing fungi

Fungal metabolism processes an apparently endless diversity of organic compounds, which are not obviously required for normal growth and metabolism: these are called

secondary metabolites. Not all secondary metabolites are mycotoxins. Simplistically, we could split them into three broad groups, being 1) toxic to bacteria (antibiotics); 2) toxic to plants (phytotoxins) and 3) toxic to animals (mycotoxins).

The exact process of mycotoxin production by fungi is not well understood. However, It is hypothesized that produced mycotoxins may be a strategy to out-compete another microorganism for plant nutrients (Rankin and Grau, 2002). Recently, Khaneghal *et al*. (2018), studied the effect of DON on the host plant. The author found that DON production prevents the formation of a thick cell wall, which facilitates fungal infection.

The most studied mycotoxins (DON, AF's, FUM, ZEN and OTA) are produced by just a few species from the common genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Claviceps*. These species can be simplistically categorized as field moulds (*Fusarium*, and *Claviceps*), which infect crops as parasites, and storage fungi (*Aspergillu*s and *Penicillium*), which grow in feedstuffs stored under sub-optimal conditions. The main factor that allows this simple classification is the moisture levels of the host. Field fungi such as *Fusarium* spp. generally, require higher moisture levels (> 0.9 water activity) to grow and produce mycotoxins. Therefore, they mainly infect seeds and plants in the field. Storage fungi such as *Aspergillus* spp. and *Penicillium* spp. require lower water activity, and are thus more prominent after harvest and during storage. *Claviceps* spp. are plant pathogens that replace plant structures such as grain kernels with hardened fungal tissues called ergots or sclerotia and therefore its mycotoxins only occur in field (Tudzynski *et al.*, 2001). All *Aspergillus* spp. and *Penicillium* spp. species either are commensals, growing in crops without obvious signs of pathogenicity, or invade crops after harvest and produce toxins during drying and storage. The most important *Aspergillus* species, occurring in warmer climates, are *A. flavus* and *A. parasiticus*, which produce AF's in maize, groundnuts, tree nuts, and, less frequently, other commodities. *Penicillium verrucosum* also produces ochratoxin A, but occurs only in cool temperate climates, where it infects small grains. *Fusarium verticillioides* is ubiquitous in maize, with an endophytic nature, and produces FUM, which are generally more prevalent when crops are under drought stress or suffer excessive insect damage. *F. graminearum*, which is the major producer of DON and ZEN, is pathogenic on maize, wheat, and barley, and produces these toxins whenever it infects these grains before harvest.

1.2.2 Conditions for fungal growth and mycotoxin production

1.2.2.1 Fungal biological requirements

Fungal growth and consequence occurrence of individual mycotoxins it is a result of complex interaction of several factors, being environmental conditions (geography and climatic factors) the most relevant (Kuiper-Goodman, 2004; Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011; Ramirez *et al.*, 2006). Behind these two main factors (geography and climatic factors), biological requirements namely temperature and water activity (Table S1.3) will play a critical role on fungal growth and consequent mycotoxin production (CAST, 2003; FAO, 2004; Marth, 1992; Ramirez *et al.*, 2006; Sweeney and Dobson, 1998). However, fungal growth and mycotoxin production are not simple combinations of optimal temperature and water activity. Ramirez *et al*. (2004) observed that the type of water stress, whether caused by osmotic or matric forces, also impacts on the activity and colonisation of cereal-based substrates by strains of *F. graminearum* (the main producer of DON). Several studies on *F. graminearum* from root and stalk rot of cereals have shown optimum water content and temperature for growth were 0.99–0.98 aw at 20–30 °C changing to 0.95–0.96 aw at 35 °C. However, conditions for optimum fungal growth and maximum toxin production are not the same (Ramirez *et al.*, 2006).

1.2.2.2 Deoxynivalenol: its structure and production

Trichothecenes are a family of more than 200 structurally related compounds (Haschek and Beasley, 2009). The structure of trichothecenes is characterized by a sesquiterpene ring and a C-12, 13-epoxide ring (Figure 1.1). They are mainly produced by several *Fusarium* species (e.g. *F. sporotrichioides, F. graminearum, F. poae*, and *F. culmorum*), but can also be produced by members of other genera such as *Myrothecium* (Tamm and Breitenstein, 1984) and *Trichothecium* (Jones and Lowe, 1960). Trichothecenes include T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (known as DON or vomitoxin), and nivalenol (NIV). Both T-2 toxin and DAS are the most toxic and are soluble in non-polar solvents (e.g. ethyl acetate and diethyl ether) whereas DON and its parent compound NIV are soluble in polar solvents such as alcohols (Hussein and Brasel, 2001; Trenholm *et al.*, 1986). The trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with initiation, elongation, and termination stages (Kumar *et al.*, 2013). Deoxynivalenol is the most frequently occurring trichothecene mycotoxin, for the time being, and frequently found in cereal grains worldwide (Gonçalves *et al.*, 2018b;

Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Rodrigues and Naehrer, 2012). As *Fusarium* spp. are highly influenced by environmental conditions, an increase in its occurrence has been speculated due to climate change with possible DON production increase (Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011).

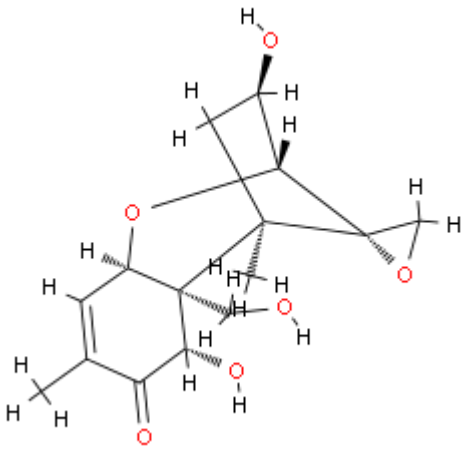

Figure 1.1: Chemical structure of Deoxynivalenol, IUPAC name (3β,7α)-3,7,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one. Molecular formula $C_{15}H_{20}$. Source: ChemSpider ref.:36584.

1.2.3 Contamination of crops and feeds

Presence of moulds in crops/ raw materials or finished feeds, may be the first indication that something is incorrect with its hygiene/ conservation, however, it will be impossible to correlate the fungal presence with the potential presence of the mycotoxin and vice-versa (Alinezhad *et al.*, 2011; Greco *et al.*, 2015). Extending the knowledge of fungal contamination in crops, practically there are several reasons why finished feeds may get mouldy, from improper storage conditions (high humidity, high variations in temperatures leading to condensation, etc.) to the poor manufacturing process (e.g., insufficient drying time, lack of preservatives/anti-moulds, etc.). Fungi contamination can also originate from an inappropriate selection of ingredients, which can carry fungi spores that are resistant to extrusion/pelleting, having the capacity to germinate afterwards (due to improper storage or poor manufacturing processes). The presence of fungi might also be a direct risk for the host, e.g., *Fusarium oxysporum* and *Fusarium solani*, known as opportunistic pathogens for fish and shrimp (Hatai *et al.*, 1986; Lightner, 1996; Ostland V.E. *et al.*, 1987; Souheil *et al.*, 1999). Fungi presence in feeds may also reduce the

palatability and therefore intake of the feed, however, its presence cannot be correlated with the presence of the mycotoxin and vice-versa (Alinezhad *et al.*, 2011; Greco *et al.*, 2015).

Moreover, mycotoxins produced on crops in the field (which is the case of DON) will remain in raw materials, even after processing, due to their heat stability (Pitt, 2014), while fungi will be destroyed due to high temperatures. For example, *Fusarium* spp. are field fungi usually lacking the ability to grow on dry feed. However, the toxins produced by these fungi species (e.g., DON) will remain stable on the plant raw materials used to manufacture aquafeeds, and in some cases, even be redistributed and concentrated in certain milling fractions (Cheli *et al.*, 2013) e.g., corn vs corn gluten meal (Gonçalves *et al.*, 2018b). Mycotoxin redistribution and transfer from crops to aquafeeds have been observed and reported (Cheli *et al.*, 2013; Gonçalves *et al.*, 2018b).

1.3 Consequences of mycotoxins for aquaculture

1.3.1 Mycotoxin occurrence in aquaculture feeds

For most aquaculture species, the selection of plant proteins is based on a combination of local market availability, cost and the nutritional profile (including anti-nutritional factor content and level) of the protein meal in question (Davis and Sookying, 2009; Gatlin *et al.*, 2007; Krogdahl *et al.*, 2010). However, evaluating mycotoxin contamination is not common practice, at least for *Fusarium* spp. mycotoxins (i.e., DON, FUM, and ZEN), being most commonly analysed AF's. Moreover, as clinical signs are normally subclinical (see Chapter 1.3.2 for more details), mycotoxins may represent a camouflaged problem, which can lead to increased disease susceptibility and poor performance. With the overall increase of mycotoxin contamination in plant ingredients due to climate change (Paterson and Lima, 2010; Paterson and Lima, 2011) and the simultaneous increase of plant meals for aquafeeds, it is important to understand the occurrence levels and type of mycotoxins in plant meals commonly used in aquafeeds.

Peer-reviewed literature on the occurrence of mycotoxins in aquafeeds is not as common as for livestock industry; however, there is already a considerable amount of references (Table 1.1), which helps to understand the potential risk of mycotoxins in aquafeeds. The present thesis will be mostly focused on the occurrence of DON in aquafeeds. However, a critical overview of existing literature on this topic may be consulted in the original publication, which this chapter is based in Gonçalves *et al*. 2020.

1.3.1.1 Limitations of mycotoxin occurrence studies

Analysing the peer-reviewed literature on the occurrence of mycotoxins in aquafeeds, summarized in Table 1.1, it is possible to notice a pattern on the target mycotoxins analysed in the feed. In samples analysed before 2012, the main mycotoxins analysed were AF's (AFB_1, AFB_2, AFG_1, AFG_2; in most of the cases only AFB_1; see Table 1.1) and in some cases ZEN and OTA (Fegan and Spring, 2007) (with the exception of Martins *et al.*, 2008) and, possibly influenced by previous data reported on terrestrial livestock feed samples. After 2012, other mycotoxins were beginning to be reported besides AF's (Table 1.1). These studies have either targeted the analysis of specific mycotoxins due to the inclusion of certain plant meals (e.g., Pietsch *et al.*, 2013; Woźny *et al.*, 2013) or explored a broad mycotoxin occurrence (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Nácher-Mestre *et al.*, 2015). This different pattern in the target mycotoxin analysed in feed might have different justifications. The screen of a higher number of mycotoxin in samples after 2012, might be a reflection of increasing awareness of mycotoxins in aquaculture feeds. However, we cannot exclude also, the easier access to analytical instrumentation to determine mycotoxins together with the evolution of the analytical methods *per se* as a plausible contribution to this change.

Independent of the reason that originated this pattern, on samples preceding 2012 it is impossible to evaluate the risk of DON, as this mycotoxin, as well as other *Fusarium* spp. mycotoxins were generally not screened.

1.3.1.2 Aquafeed samples preceding 2012

The oldest documented survey of mycotoxins occurrence in aquaculture finished feed is from Bautista *et al*. (1994). In this study, a total of 62 samples collected in the Philippines between August 1990 to February 1991. Samples were black tiger shrimp (*Penaeus monodon*) feeds, sourced from feed mills and at farm level (Table 1.1). Other studies also focused its target on AF's analysis, which was the case of Bintvihok *et al*. (2003) which analysed samples collected in the eastern and southern regions of Thailand (1997 to 1998) and by Altuğ and Berklevik (2001) with samples collected in Turkey from 1998 to 2000 (Table 1.1). In 2007, Fegan and Spring (2007) reported, to our knowledge, the first and most complete mycotoxin occurrence survey on fish and shrimp feeds before 2012 and for the first time considering the presence of *Fusarium* spp. mycotoxins on its samples. Samples were collected in India and Thailand and analysed for the presence of AF's, T-2,

ZEN and OTA. No information is available on the period of sampling, region area or sample origin (feed mill or farm). Nonetheless, the information reported shows a different contamination pattern between fish and shrimp feeds and shows co-occurrence of mycotoxins. Out of the nine fish feed samples analysed from Thailand, all samples were contaminated predominantly by ZEN, at levels ranging from 36.20 to 118.48 µg kg $^{-1}$, followed by T-2 (2.6 to 50.03 µg kg $^{-1}$) and OTA (2.32 to 7.74 µg kg $^{-1}$). Also in Thailand, shrimp feed samples (n = 7) were contaminated with ZEN and OTA while no data on AF's was available (Table 1.1). Shrimp feed samples (n = 10) collected from India were mostly contaminated with AF's, ranging between 40 and 90 µg kg $^{-1}$. In this study, Fegan and Spring (2007) also reported mycotoxin occurrence in the raw materials used to formulate aquafeeds, highlighting the occurrence of mycotoxins (T-2 and ZEN and OTA) in marine ingredients (fishmeal from China, Myanmar, Thailand; fish and shrimp meal from Thailand) which will not be further discussed in this thesis, however, is deeper explored in Gonçalves *et al.* (2020).

An exception to the almost exclusive AF's analysis in finished feeds prior to 2012, are the results presented by Martins *et al*. (2008), who analysed 20 samples of fish feed sourced from Portugal for the presence of AFB_1, OTA, DON, ZEN and FB_1. In this study, no detectable levels of the target mycotoxins were obtained. In the remaining studies shown in Table 1, in which samples were collected in or before 2012 (Alinezhad *et al.*, 2011; Almeida *et al.*, 2011; Gonçalves-Nunes *et al.*, 2015), the target mycotoxin analysed in feed was always AFB_1.

1.3.1.2 Aquafeed samples after 2012

From 2012 onwards, the number of peer-reviewed publications related to the presence of mycotoxins (including not only AF's) in aquaculture feeds increased considerably. In 2013, Woźny *et al*. (2013) analysed the presence of ZEN in trout feed collected from three farms in November. One of the farms had no detected levels of ZEN while the other two farms had 81.8 ± 25.8 and 10.3 ± 0.9 µg kg $^{-1}$ of ZEN in their feed, respectively. The same study also explored the carry-over of ZEN from the feed by analysing several rainbow trout (*Oncorhynchus mykiss*) organs for ZEN presence, results that are further explored in Chapter 1.3.4. Pietsch *et al*. (2013), unveiled the presence of DON (236.18 µg kg $^{-1}$) and ZEN (63.82 µg kg $^{-1}$) in common carp (*Cyprinus carpio*) feeds in samples from central Europe. Still, in Europe, Nácher-Mestre *et al*. (2015), investigated the occurrence of mycotoxins in Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*)

feeds, with respectively, high and low inclusion of plant meals. From the 18 mycotoxins analysed, the most representative mycotoxins found were FUM and DON. In Atlantic salmon, from the three types of feeds analysed, levels of DON were 22.4, 19.4 and 23.1 µg kg $^{-1}$ and 148, 754 and 112 µg kg $^{-1}$ of FUM respectively. For gilthead sea bream, two samples were found to contain 79.2 and 53.5 µg kg $^{-1}$ of DON, and 6.4 µg kg $^{-1}$ of FUM in only one of the samples. In Argentina, Greco *et al*. (2015) also analysed salmonids feeds. In this study, 28 samples of rainbow trout (*Oncorhynchus mykiss*) feed were sampled at the farms, ranging throughout the feed portfolio for different development stages (starter feed (13 samples); grower feed (13 samples); 4 pigmented and 9 unpigmented feed and finisher feed (2 pigmented samples). The authors observed median values of: AF's = 2.82; OTA = 5.26; T-2 = 70.08; DON = 230 and ZEN = 87.97 µg kg $^{-1}$. It was also highlighted that, there was a co-occurrence of at least two out of six mycotoxins in 93% (26/28) of the analysed samples. Gonçalves *et al*. (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017) focused on unveiling the mycotoxin occurrence in plant meals (not reported here) and aquaculture finished feeds in Europe and Southeast Asia. In 2014, from January to December, 41 fish and shrimp feed samples were collected from Europe (n = 6 to 10; Croatia and Portugal) and SE Asia (n = 31; Singapore, India, Thailand and Myanmar). Samples were analysed for AF's, ZEN, DON, FUM and OTA (Table 1.1). Interestingly, a higher occurrence of FUM was found in European samples (average 3,419.92 and maximum 7,533.61 µg kg $^{-1}$) compared to SE Asia. The remaining mycotoxins showed similar occurrence average and maximum levels for Europe and SE Asia, with mycotoxins being detected in all analysed samples. In this mycotoxin survey (Gonçalves *et al.*, 2018d), it was reported that in Europe, 50% of the samples had more than one mycotoxin per sample, and in Asia, 84% of the samples were contaminated with more than one mycotoxin per feed sample.

In 2015, analysing the same mycotoxins as in the previous study, Gonçalves *et al*. (2017) sourced 25 samples of fish and shrimp feeds in Europe (n = 4; Denmark, Austria, Netherlands and Germany) and SE Asia (n = 21; Vietnam, Indonesia, Myanmar). Contrary to samples collected in 2014, the European samples analysed in 2015 showed relatively low mycotoxin contamination, with only DON contamination reaching values up to 20 µg kg $^{-1}$. In SE Asian samples, contamination was also generally lower when compared to the previous year, with only AF's showing similar contamination levels to

2014 (average contamination of 58 µg kg $^{-1}$ and maximum of 201 µg kg $^{-1}$). However, the co-occurrence risk increased in both regions.

From January to December 2016, Gonçalves *et al*. (2018d) sampled four shrimp feeds from India and 12 fish feeds from Indonesia, Myanmar, Taiwan and Thailand. Interestingly, the fish and shrimp feeds showed a relatively different mycotoxin contamination pattern, possibly due to the type of raw materials used to manufacture these diets. Fish feed samples showed lower contamination (Table 1.1) when compared with shrimp feeds. However, a higher number of co-occurring mycotoxins were observed in fish feeds. Shrimp feeds showed relatively high contamination of DON, with an average contamination level of 881.66 and maximum of 2,287 µg kg $^{-1}$.

Mycotoxins also represent a big challenge to the increasingly successful aquaculture sector on the African continent. Marijani *et al*. (2017), analysed mycotoxin occurrence in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) feeds, gathering 16 samples from Kisumu, Kenya, 13 samples from Ukerewe, Tanzania, 10 samples from Kigembe, Rwanda and 13 samples from Jinja, Uganda. Samples were collected from farms (farm-made feeds; n = 14), local feed millers (n = 14) or imported feeds from Israel and India (n = 12). From the 52 samples analysed, Marijani *et al*. (2017) observed that farm-made feeds were highly contaminated with AF's, FUM and DON (Table 1.1). On the other hand, feed samples from local feed millers, as well as the imported feed samples, had only minor contamination of AF's.

1.3.1.3 Additional remarks on the occurrence of mycotoxins in aquafeeds

The results of the most recent mycotoxin occurrence surveys of aquaculture feeds (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Marijani *et al.*, 2017; Nácher-Mestre *et al.*, 2015) clearly show an increase in mycotoxin occurrence compared to previous surveys (Alinezhad *et al.*, 2011; Almeida *et al.*, 2011; Altuğ and Berklevik, 2001; Bintvihok *et al.*, 2003). Unfortunately, it cannot be concluded, from this data, that there is a higher mycotoxin risk now compared to the past. This is because the target mycotoxins analysed in older studies were not the same and sensitivity detection levels and methodologies have since improved significantly. Nonetheless, it was theoretically expected that an increasing level of plant meals in aquafeeds would lead to increased occurrence of mycotoxins in these feeds, which is observable by the most

recent occurrence surveys (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Marijani *et al.*, 2017; Nácher-Mestre *et al.*, 2015).

Besides the increasing mycotoxin occurrence and the focus on a broad range of mycotoxins, several other important conclusions can be taken from the studies summarized in Table 1.1. A key aspect is the regional differences in mycotoxin occurrence reported and the correlation between fungi contamination and the presence of mycotoxins. The presence of moulds in a fish feed is the first indication that something is wrong with its hygiene. There are several reasons why feeds get mouldy, from improper storage conditions (high humidity, high variations in temperatures leading to condensation, etc) to the poor manufacturing process (e.g., insufficient drying time, lack of preservatives/anti-moulds, etc). Fungi contamination can also originate from an inappropriate selection of ingredients, which can carry fungi spores that are resistant to extrusion/pelleting, having the capacity to germinate afterwards (due to improper storage or poor manufacturing processes).

The regional differences in mycotoxin occurrence is also an important factor which cannot be overlooked. Fungal growth and consequently mycotoxin production in crops is influenced by several factors, with weather conditions being the most important (Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011). Consequently, it could be expected that different regions present differences in mycotoxin contamination patterns, and even within a region, mycotoxin occurrence may vary depending on seasonal conditions. This is shown by the data reported by Bintvihok *et al*. (2003) in samples from Thailand, which suggests that rainy seasons might be more problematic and therefore should be closely monitored. However, factors such as climate change and the world trade of commodities make it challenging to estimate the risk of mycotoxins in aquaculture finished feeds. For example, as reported by Gonçalves *et al*. (2018d), higher levels of FUM in European finished feeds compared to SE Asia samples cannot be easily explained and therefore a better understanding on the origin of sourced ingredients is necessary. The increasing globalisation of trade commodities and incorporation of imported raw materials into aquafeeds exposes the industry to the potential risk of mycotoxins, which are sometimes not even common for the region (not the case in that particular study). Therefore, mycotoxin contamination needs to take into account the globalisation of raw materials.

Table 1.1: Documented mycotoxin occurrence in aquaculture feeds.

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (µg kg $^{-1}$)	Method of analysis	Observations
Aquafeed samples preceding 2012									
Bautista *et al.* 1994	August 1990 - February 1991 (rainy season)	Philippines	Feed plant Farm level	n = 62	Black tiger shrimp (*Penaeus monodon Fabricius*)	AFB_1	n = 2 -> none detected n = 36 -> 10 to 20 µg kg $^{-1}$ AFB_1 n = 21 -> 30 to 40 µg kg $^{-1}$ AFB_1 n = 2 -> 60 to 120 µg kg $^{-1}$ AFB_1	HPTLC	------
Bintvihok *et al.* 2003	[S]Summer (March - June 1997) [R]Rainy (July - October 1997) [W]Winter (November - February 1998)	Thailand (Eastern and Southern regions)	Farm level	N_t =150 (50 samples from 10 different regions during 3 seasons)	Black tiger shrimp (*Penaeus monodon Fabricius*)	AFB_1 AFB_2 AFG_1 AFG_2	Eastern region [S]0.003–0.012 [R]0.003-0.651 [W]0.003-0.314 Southern region [S]0.004 [R]0.003-0.058 [W]0.003-0.022	HPLC	▪ Feeds composed mainly of fishmeal, soybean and corn (no information on ingredient inclusion levels or finished feed storage period)
Altuğ *et al.* 2001	1998, 1999, 2000	Turkey	Farm level Feed plant Imported feeds	n = 170	Rainbow trout Seabream Pike-perch	AFB_1	n = 20 > 20 µg kg $^{-1}$ n = 85 = 21.2 to 42.4 µg kg $^{-1}$ n = 22 = 5.0 to 20.0 µg kg $^{-1}$ n = 43 < LOD	TLC ELISA	▪ Level of aflatoxins were higher in samples that were taken from farm level compared to feed plant or imported feed samples
Alinezhad *et al.* 2011	March - July 2009 (1 sample per month)	Iran	Feed plant	n = 6	Rainbow trout	AFB_1	0.12 to 20.09 µg kg $^{-1}$ AFB_1	HPLC	▪ High concentrations of AFB_1 in fishmeal ($\bar{x}$= 67.35 µg kg $^{-1}$) and soybean meal ($\bar{x}$= 30.88 µg kg $^{-1}$)
Fegan & Spring, 2007	n/a	[IN]India [TH]Thailand	n/a	[IN,S]n= 10 [TH,S]n= 7 [TH F]n= 9	Shrimp[S] Fish[F]	[IN,S](AF, T-2, ZEN) [TH,S](T-2, ZEN, OTA) [TH,F](T-2, ZEN, OTA)	[IN,S]AF = 40-90; (9/10) [IN,S]T-2 = 20-40; (4/10) [IN,S]ZEN = 20-40; (4/10) [TH,S]T-2 = 2.6-50.03; (3/7) [TH,S]ZEN = 16.78-23.00; (6/7) [TH,S]OTA = 2.32-7.74; (7/7) [TH,F]T-2 = 15.91-49.13; (9/9) [TH,F]ZEN = 36.20-118.48; (9/9) [TH,F]OTA = 2.16-9.72; (9/9)	n/a	▪ Marine ingredients (fishmeal from China, Myanmar, Thailand; fish and shrimp meal from Thailand) contaminated with T-2, ZEN and OTA
Goncalves-nunes *et al.* 2015	January - March 2009	Brazil (Piauí State)	Feed plant	n = 18	Fish	AFB_1	1.6 - 9.8	ELISA	▪ Finished feed samples were composed of soybean bran (15%), corn bran (27%), other cereals (57.5%).
Barbosa *et*	September 2009	Brasil	n/a	n = 60	n/a		FB_1 = (90%) 0.3-4.94; $\bar{x}$ = 2.6	FB_1 -	LOD:

***al*. 2013**	and August 2010	(Rio de Janeiro State)				FB_1 AFB_1 OTA	AFB_1 = present in 55% of the samples OTA = present in 3.3% of the samples No levels mentioned for AFB_1 and OTA	ELISA AFB_1 and OTA - TLC	▪ 0.2 µg g^{-1} for ELISA (FB_1) ▪ 0.003 and 0.005 µg g^{-1} for TLC (AFB_1 and OTA) ▪ 50% of samples had co-occurrence of AFB_1 and FB_1 ▪ 3.3% of the samples tested positive for the three mycotoxins analysed
Martins *et al*. 2008	n/a	Portugal	n/a	n = 20	Fish	AFB_1 OTA DON ZEN FB_1	N.d	HPLC	LOD ▪ AFB_1 = 0.2 µg kg^{-1} ▪ OTA = 20 µg kg^{-1} ▪ DON = 100 µg kg^{-1} ▪ ZEN = 50 µg kg^{-1} ▪ FUM = 20 µg kg^{-1}
Almeida *et al*. 2011	n/a	Portugal	Feed plant	n = 87	Seabass	AFB_1	AFB_1 n.d. (detection limit of the method was 1.0 µg kg^{-1})	HPLC	▪ 35 samples contaminated with *Aspergillus* spp.
Aquafeed samples after 2012									
Pietsch *et al*. 2013	n/a	Central Europe	n/a	n = 11	Carp	DON ZEN	DON = 66-825; $\bar{x}$ = 236.18 ZEN = 3-511; $\bar{x}$ = 63.82	HPLC	▪ Most common plant ingredients in feeds collected: C = corn; CGF = Corn gluten feed; SEM = soybean extraction meal; SM = soybean meal; SFEM = sunflower feed extraction meal; W = wheat; WB = wheat bran, WDB = wheat distillery by-product; WGF = wheat gluten feed.
Woźny *et al*. 2013	November 2012	Poland (North-eastern region)	Farm level	n = 3	Trout	ZEN	$\#_1$ = n.d. $\#_2$ = 81.8 ± 25.8 $\#_3$ = 10.3 ± 0.9	HPLC	▪ Rainbow trout organs were also sampled, refer to table 6.
Greco *et al*. 2015	n/a	Argentina (Río Negro and Neuquén)	Farm level	n =28	Rainbow trout	AF OTA T-2 FUM DON ZEN	AF = 1.3 – 8.91; $\tilde{x}$ = 2.82 OTA = 3.5 – 5.0 $\tilde{x}$ = 5.26 T-2 = 50 – 105.99; $\tilde{x}$ =70.08 FUM = 190 -222; $\tilde{x}$ = -- DON = 150 – 210; $\tilde{x}$ =230 ZEN = 20.04 – 159.76; $\tilde{x}$ = 87.97	ELISA	▪ Finished feed samples were composed of soybean expeller, disabled soybean, corn, wheat, wheat bran, corn gluten meal ▪ Co-occurrence of at least two out of six mycotoxins was recorded in 93% (26/28) of samples analysed
Nacher-Mestre *et al*. 2015	n/a	United Kingdom	Feed plant	n = 5 2 diets[GSB] with low level plant meal 3 diets[AS] with high level plant	[AS]Atlantic salmon [GSB]Gilthead sea bream	AFB_1, AFB_2, AFG_1, AFG_2, OTA, NEO, FB_1, FB_2, FB_3, T-2, DIA, ZEN, NIV, DON, 3-AcDON, 15-AcDON, FUX, and HT-2	DON^{GSB} = 79.2 and 53.5 DON^{AS} = 22.4 , 19.4 and 23.1 FUM^{GSB} = -, 6.4 FUM^{AS} = 148, 754 and 112	LC–MS/MS	▪ No carry-over effects observed after 8^{GSB} and 7^{AS} months of feeding the contaminated diets. ▪ Diets manufactured with contaminated ingredients (wheat (n = 3, Germany and Denmark), wheat gluten (n = 4, UK, Germany, and China), pea (n = 1, Denmark), pea

				meal					protein (n = 2, Norway), rapeseed meal (n = 1, Denmark), corn gluten (n = 3, China and Germany), soya protein (n = 4, Brazil) and sunflower meal (n = 1, Russia).
Gonçalves *et al*. 2016	January 2014 – December 2014	[A]Asia (CN, IN, TH, MN) [E]Europe (CR, PT)	Farm level Feed plant	N_t= 41 samples n = 31 Asia n = 6-10 Europe	Shrimp Fish	AF ZEN DON FUM OTA	[A]AF: x̄ = 51.83; Max = 220.61; (21/31) [A]ZEN: x̄ = 60.41; Max = 232.88; (18/31) [A]DON: x̄ = 160.86; Max = 413.08; (21/31) [A]FUM: x̄ = 172.63; Max = 573.32; (18/31) [A]OTA: x̄ = 2.11; Max = 5.05; (17/31) [E]AF: x̄ = 0.43; Max = 0.43; (1/6) [E]ZEN: x̄ = 118.01; Max = 305.89; (4/6) [E]DON: x̄ = 165.61; Max = 281.72 (4/6) [E]FUM: x̄ = 3419.92; Max = 7533.61; (3/10) [E]OTA: x̄ = 1.53; Max = 3.1; (4/6)	HPLC	▪ In Europe, 50% of the samples had more than 1 mycotoxin per sample ▪ In Asia, 84% of the samples had more than 1 toxin per feed
Gonçalves *et al*. 2017	January – December 2015	[A]Asia (VN, ID, MM) [E]Europe (DK, AT, NL, DE)	Farm level Feed plant	N_t= 25 [A]n= 21 (20/21) [E]n= 4 (4/4)	Shrimp Fish	AF ZEN DON FUM OTA	[A]AF: x̄ = 58; Max = 201 [A]ZEN: x̄ = 53; Max = 157 [A]DON: x̄ = 29; Max = 63 [A]FUM: x̄ = 58; Max= 238 [A]OTA: x̄ = - ; Max = 7 [E]AF: not detected [E]ZEN: x̄ = -; Max = 6 [E]DON: x̄ = -; Max = 20 [E]FUM: n.d. [E]OTA: n.d.	HPLC	------
Marijani *et al*. 2017	n/a	Kenya Kisumu -> n = 16 Tanzania Ukerewe -> n = 13 Rwanda Kigembe -> n = 10 Uganda Jinja -> n = 13	[FM]Farm [LFP]Local feed plant [IF]Imported feed (from Israel and India) [FI]Feed Ingredients	N_t=52 [FM]n= 14 [LFP]n = 14 [IF]n = 12 [FI]n = 12	Nile tilapia African catfish	3-ADON 15-ADON DON AF DAS AOH FB_1 FB_3 OTA ROQ-C	[FM]AF = 2.4-126; x̄ = 71.0 ± 31.5 [FM]FUM = 33.2-2834.6; x̄ = 1136.5 ± 717.9 [FM]DON = 69.1-755.4; x̄ = 245.8 ± 190.1 [LFM]AF = <2-28; x̄ = 11.6 ± 0.7 [LFM]FUM, DON = <LOD [IF]AF = <2-2.6; x̄ = 1.4 ± 0.9 [IF]FUM, DON = <LOD	LC–MS/MS	▪ Farmers who formulate their own feed used: sunflower seed cake, rice bran, cotton seed cake, maize bran and soybean. ▪ Feeds co-contaminated with 12[FM], 4[LFM] and 5[IF] mycotoxins. ▪ NEO, FUX and STERIG were not detected in any of the samples ▪ AF co-occurred with FUM in 13 of 24 feed samples ▪ DON co-occurred with FUM in 2 of

									24 feed samples
Gonçalves *et al*. 2018	January – December 2016	Asia (SAS: IN, ID, MN, TW, TH)	Farm level Feed plant	N_t= 16 ^{S}n= 4 ^{F}n= 12	Shrimp^{S} Fish^{F}	AF ZEN DON FUM OTA NIV 3-AcDON 15-AcDON FUX T-2 HT-2 DAS NEO	^{F}AF: $\bar{x}$ = 51.83; Max = 32; (8/12) ^{F}ZEN: $\bar{x}$ = 75.66; Max = 153; (6/12) ^{F}DON: $\bar{x}$ = 82.87; Max = 396; (8/12) ^{F}FUM: $\bar{x}$ = 354.22; Max = 993; (9/12) ^{F}OTA: $\bar{x}$ = 1.65; Max = 3; (6/12) ^{S}AF: $\bar{x}$ = 0.43; Max = 24; (4/4) ^{S}ZEN: $\bar{x}$ = 22.0; Max = 53; (3/4) ^{S}DON: $\bar{x}$ = 881.66; Max = 2287 (3/4) ^{S}FUM: $\bar{x}$ = - ; Max = 43; (1/4) ^{S}OTA: $\bar{x}$ = 2.66; Max = 4; (3/4)	LC-MS/MS	------

Reference entries are in chronological ordered by sampling date collection or publishing date. Superscript letters give extra information; they are only valid for the same row.
General abbreviations: $\bar{x}$ = average value; $\tilde{x}$ = median value; Max = maximum; HPLC = High-performance liquid chromatography; ELISA = enzyme linked immunosorbent assay; LC–MS/MS = Liquid chromatography-tandem mass spectrometry; TLC = Thin layer chromatography; HPTLC = high performance thin layer chromatography ; LOD = limit of detection; n.d.= not detected
Mycotoxins: AF: aflatoxins (the sum of AFB_1, AFB_2, AFG_1 and AFG_2); AFB_1= aflatoxin B_1; AFB_2= aflatoxin B_2; AFG_1= aflatoxin G_1; AFG_2= aflatoxin G_2; DON = deoxynivalenol; FUM = fumonisins (the sum of FB_1 and FB_2); FB_1= fumonisin B_1; FB_2= fumonisin B_2; OTA= ochratoxin A; ZEA= zearalenone; NIV= Nivalenol; 3-AcDON= 3-Acetyldeoxynivalenol; 15-AcDON= 15-Acetyldeoxynivalenol; FUX= fusarenon X-glucoside; fumonisins; DAS= Diacetoxyscirpenol; NEO= neosolaniol; AOH= alternariol; ROQ-C= roquefortine C; STERIG= sterigmatocystin.
Regions: NAS = northern Asia; SAS = South-East Asia; CN = China; IN = India; TH = Thailand; MN = Myanmar; ID = Indonesia; TW = Taiwan; HR = Croatia; PT = Portugal; DK = Denmark; AT = Austria; NL = the Netherlands; DE = Germany.

1.3.2 Impact of mycotoxins in aquaculture species

Research characterizing the adverse effect of mycotoxins on the performance and health of animals has in large part focused on terrestrial livestock species (D'Mello and Macdonald, 1997; Pestka, 2007; Rotter *et al.*, 1996). However, since an aflatoxicosis outbreak in trout in the 1960s (Wolf and Jackson, 1963), research has also been carried out on the effects of mycotoxins in aquaculture species. This line of research became even more important in recent years with the increasing inclusion levels of plant meals in aquafeeds (Chapter 1.3.1; (Anater *et al.*, 2016; Goncalves *et al.*, 2018; Gonçalves *et al.*, 2018a; Hooft and Bureau, 2017; Hooft *et al.*, 2011; Wang *et al.*, 2016).

The effects of mycotoxins in fish and shrimp are diverse, varying from immunosuppression to death, depending on toxin-related (type of mycotoxin consumed, level and duration of intake), animal-related (animal species, sex, age, general health, immune status, nutritional standing) and environmental-related (farm management, biosecurity, hygiene, temperature) factors. Therefore, it is often difficult to trace observed problems back to mycotoxins. Several scientific publications report the effects of mycotoxins in fish or shrimp at different contamination levels, enabling a better understanding of mycotoxin-related ailments (Table 1.2 and supplementary tables S1.4 to S1.11). However, there are still only a few validated clinical symptoms of mycotoxin exposure in fish and shrimp. The majority of the described effects of mycotoxins in fish and shrimp (see a review from (Anater *et al.*, 2016)), are general symptoms and could be attributed to diverse pathologies or challenges, *e.g.* anti-nutritional factors or lectins in the diet (Hart *et al.*, 2010). Two notable exceptions are aflatoxicosis (yellowing of the body surface (Deng *et al.*, 2010)) and increase of the sphinganine to sphingosine ratio due to ingestion of FUM (Tuan *et al.*, 2003). The most frequently reported clinical manifestations of mycotoxin ingestion are a reduction in growth performance, alteration of haematological (erythrocyte/leucocyte count) or biochemical (alanine aminotransferase (ALT), aspartate transaminase (AST) or alkaline phosphatase (ALP)) blood parameters, liver alterations or the suppression of immune parameters.

This thesis will only focus on the impact of DON in aquaculture species, however, a broader overview of existing literature for other mycotoxins (AF's, FUM, OTA and ZEN/T-2) may be consulted in the supplementary material (AF's – fish - S1.4/ shrimp S1.5; FUM – fish – S1.6/ shrimp – S1.7; OTA – fish - S1.8 / shrimp – S1.9; ZEN/T-2 – fish-S1.10/ shrimp S1.11).

1.3.2.1 Impact of deoxynivalenol in aquaculture species

In aquaculture species, DON ingestion has been associated with a highly significant decrease in growth, feed intake, feed efficiency and protein and energy utilization (Gonçalves *et al.*, 2018c; Gonçalves *et al.*, 2018e; Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Matejova *et al.*, 2015; Ryerse *et al.*, 2015; Table 1.2). One of the most sensitive species to low levels of DON is rainbow trout (*Oncorhynchus mykiss*) (Gonçalves *et al.*, 2018c; Gonçalves *et al.*, 2018e; Hooft *et al.*, 2011; Woodward *et al.*, 1983). Hooft *et al.* (2011) reported that low, graded levels of DON ranging from 300 to 2,600 µg kg $^{-1}$ feed caused a highly significant decrease in growth (- 40%), feed intake (- 52.7%), feed efficiency (- 76.7%) and protein and energy utilization (- 74.4% and - 72.1%) when compared to the control group that received uncontaminated feed. Recently, Gonçalves *et al.* (2018e) observed that in *O. mykiss* (2.5 g), diets contaminated with 4,700 or 11,400 µg DON kg $^{-1}$ fed for 60 days decreased final body weight, specific growth rate, feed intake, hepatosomatic index and protein efficiency ratio. Furthermore, these fish showed an altered whole body composition and whole body nutrient retention. Pepsin activity in stomach samples and lipase activity in intestine samples was increased in fish that received 11,400 µg DON kg $^{-1}$. Furthermore, fish that received 4,700 or 11,400 µg DON kg $^{-1}$ feed showed increased mRNA expression of insulin-like growth factors 1 and 2 in the liver, and of two peptides that regulate feed intake, neuropeptide Y precursor and adenylate cyclase-activating polypeptide, in the brain. Another study with Atlantic salmon (*Salmo salar* L.) found that fish fed 3,700 µg DON kg $^{-1}$ showed a 20 % reduction in feed intake and a 31 % decrease in specific growth rate (Döll *et al.*, 2010). Channel catfish (*Ictalurus punctatus*) fed diets containing up to 10,000 µg DON kg $^{-1}$ either in purified form or contained in naturally contaminated wheat showed no differences in feed consumption, growth, haematocrit values or liver weights compared to animals that received uncontaminated feed (Manning *et al.*, 2014). A feeding trial in carp (*Cyprinus carpio* L.) using three different concentrations of DON (352, 619 and 953 µg kg $^{-1}$) showed immunosuppressive effects of low dietary DON concentrations (Pietsch *et al.*, 2014).

Regarding the impact of DON on shrimp, it was observed that DON levels ranging from 200 to 1,000 µg kg $^{-1}$ diet significantly reduced body weight and/or growth rate in Pacific white shrimp (*Litopenaeus vannamei*; Trigo-Stockli *et al.*, 2000). Also in *P. monodon*, dietary DON levels of 0.5 – 2,000 µg kg $^{-1}$ fed for 8 weeks decreased the

specific growth rate, the feeding rate, and the activity of liver enzymes in serum (Supamattaya *et al.*, 2005) (Table 1.2).

1.3.2.2 Combined effects of mycotoxins

Considering that compound feed contains a mixture of several raw materials and, adding to this, that mycotoxigenic fungi are usually capable of producing more than one mycotoxin, it is not a surprise to frequently observe mycotoxin co-occurrence in aquaculture finished feeds (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Mycotoxin co-occurrence in fish feeds was reported in the past for Egypt (Abdelhamid *et al.*, 1998), USA (Lumbertdacha *et al.*, 1995), Indonesia (Ali *et al.*, 1998), Nigeria (Omodu *et al.*, 2013), Central Europe (Pietsch *et al.*, 2013), Brasil (Hashimoto *et al.*, 2003), and generally for Southeast Asia and Europe (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Despite the fairly well-documented mycotoxin co-occurrence in aquaculture feed and the awareness that mycotoxin co-exposure may lead to additive, synergistic or antagonistic toxic effects, little is known about the real impact of multi-mycotoxin exposure in aquaculture species.

Is not the objective of this thesis to study the effects of mycotoxin multi-exposure, however, is important to highlight that some studies have shown that combined mycotoxins might lead to additive or synergistic effects in aquatic species. In table S1.12, is summarized the studies reporting mycotoxin multi-exposure in fishes (*Ictalurus punctatus*, MON:FB_1 (Yildirim *et al.*, 2000); *Clarias gariepinus*, AFB_1:FB_1 (Adeyemo *et al.*, 2018); *O. mykiss*, FB_1:AFB_1 (Carlson *et al.*, 2001); *Danio rerio*, CTN:PAT (Wu *et al.*, 2012); *Cyprinus carpio*, AFB_1:DON (He *et al.*, 2010) and AFB_1:OTA (Agouz and Anwer, 2011); *Oreochromis niloticus*, AFB_1:OTA (Mohamed *et al.*, 2010); *Gambusia affinis*, AFB_1:FB_1 (McKean *et al.*, 2006b)) and shrimp's, (*L. vannamei*, AFB1:FB1 (Pérez-Acosta *et al.*, 2016). The only study addressing the combined effects of DON and AFB_1 in primary hepatocytes (Table 1.2) (He *et al.*, 2010), concluded that the toxic effect of the combined mycotoxins was bigger than the effects of single mycotoxins. As mycotoxin co-contamination of aquaculture feed is frequently observed, combined effects of mycotoxins in aquaculture species should be more thoroughly investigated in the future.

Table 1.2: Revision of literature on the effects of deoxynivalenol (single mycotoxin and multi-exposure) on fish species and shrimp species

Species	Tested dosage (µg DON kg^{-1} feed)	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues (µg DON kg^{-1} tissue)	OBS.
Atlantic salmon *(Salmo salar)*	Control; 4x[DON] 800-3,700[1] µg kg^{-1}	Döll *et al.* 2010	Y	n/a	↓FI[1] ↓SGR[1] ↑FCR[1]	n/a	n/a	n/a	● Initial weight: 405±31 g, 15 weeks study
Atlantic salmon *(Salmo salar)*	0; 2[1] and 6[2] µg kg^{-1}	Bernhoft *et al.* 2017	n/a	n/a	↓FI[1,2]	n/a	n/a	L/M/K/SK (µg kg^{-1}) [1,t1]=12.2/5.6/n.s./n.s. [1,t2]=12.8/8.5/n.s./n.s. [1,t3]=18.1/6.0/12.3/n.s. [2,t1]=9.6/10.3/n.s./n.s. [2,t2]=20.2/17.3/n.s./n.s. [2,t3]=28.6/18.6/16.8/20.8	● Initial weight: 58 g, 8 weeks study; ● Sampling at 3[t1], 6[t2] and 8[t3] weeks ● n.s.=not sampled
Channel catfish *(Ictalurus punctatus)*	0; 3,300[1]; 5,500[2]; 7,700[3]; 8,800[4] µg kg^{-1}	Manning *et al.* 2014	n/a	n/a	↓FCR[4] N =FI =WG	n/a	Y[C] ↓M[2-4]	n/a	● Initial weight: 5.87±0.22 g, 7 weeks study ● Naturally contaminated corn (DON) mixed with clean corn ● Challenge[C] by bath with *Edwardsiella ictaluri* (21 days infection)
Common carp *(Cyprinus carpio)*	352[1], 619[2] and 953[3] µg kg^{-1}	Pietsch *et al.* 2014	n/a	n/a	=FW =FTL =CF	=Hct; Hb; SSI, WBC ↓EryL[1] =Ery.N.L ↓Ery.N.W[2] ↑SOD[1, RP] ↑CAT[1, RP]	n/a	Muscle samples [ng g^{-1} dry weight] [1]= 0.6; [1, RP]= 1.4 [1]= 1.3; [1, RP]= 0.7 [1]= 1.2; [1, RP]= 0.0	● Raised from eggs (average initial weight 36 g), 4 weeks study ● Leaching test – 50% of DON leaches in first 2h ● Additional 2 weeks of feeding uncontaminated diet – recovery period[RP]
Common carp *(Cyprinus carpio)*	352[1], 619[2] and 953[3] µg kg^{-1}	Pietsch *et al.* 2014 b	↑FA[2,3] ↑Hp[1-3] ↑Ds[1,3] [RP]↑FA[1,3] [RP]↑Hp[3] [RP]↑Ds[1] [HK]↑Lp[3] [K]↓Lp[3]	n/a	=FW =FTL =CF	[HK] ↑LDH[1-3] [K] ↑LDH[1-3] [M]↓LDH[3] [HK;RP]↑LDH[3] [K;RP]↑LDH[3] ↑ALT[2] [Se]↑Lact[3] [Se]↑LDH[3]		n/a	● Raised from eggs (average initial weight 36 g), 4 weeks study ● Additional 2 weeks of feeding uncontaminated diet – recovery period[RP]

			K; RP ↓Lp[1-3] Sp ↑Lp[3] L ↑Lp[3] L;RP ↓Lp[3] ↑PRE[3] RP ↑PRE[1-3] ↑ERE[2] RP ↑ERE[3] RP ↑ARE[3]			Se ↑AST[3] Se ↓ALT[3] Se ↓Alb[2,3] Se; RP ↑gluc[1-3] Se; RP ↑Lac[1-3] Se; RP ↑LDH[1,3] Se; RP ↑ALT[3]			
Common carp *(Cyprinus carpio)*	0; 5,960 µg DON kg $^{-1}$ + 0,330 µg 15-acetyl DON kg $^{-1}$	Pelyhe *et al.* 2016	↑GSH [t4] HP =MDA =GSH =Gpx	n/a	n/a	↑GSH [t4] ↑*gpx4a* [t3,t4] ↑*Gpx4b* [t1-t4]	Y	n/a	● Initial weight: 23.26±6.86 g, 4 weeks study ● Control diet contaminated with: T-2, <20 µg kg $^{-1}$; HT-2,<20 µg kg^{-1}; DON, <20 µg kg $^{-1}$; and 15-acetyl DON,<20 µg kg $^{-1}$ ● Sampling at 7[t1], 14[t2], 21[t3], 28[t4]
Common carp *(Cyprinus carpio)*	0 and 953 µg kg^{-1}	Pietsch *et al.* 2015	L Hp[t2] V[t2] Ds[t2, t3] Fa[t3]	n/a	n/a	↑ALT[t2,t4] ↓GSH[t1,t2] ↓PROD[t1] ↑GST[t4]	n/a	n/a	● Initial weight: 9-12 cm in length, 4 weeks study ● Sampling at 7[t1], 14[t2], 26[t3], 56[t4] ● No effects on: POD, AscPx, GPx, Se-GPx, GSH-Red, CAT, SOD
Grass carp *(Ctenopharyngodon idella)*	27; 318[1]; 636[2]; 922[3]; 1,243[4] and 1,515[5] µg kg $^{-1}$	Huang *et al.* 2018	↓ILgth[2-5] ↓ILI[2-5] ↓IW[1-5] ↓ISI[3-5] IHyp[2-5]	↑ROS[PI, MI, DI; 2-5] ↑MDA [PI, MI, DI; 2-5] ↑PC [PI, MI, DI; 2-5] ↓ASA [PI, MI, DI; 2-5] ↓AHR [PI, MI; 3-5; DI; 2-5] ↓T-AOC [PI, MI; 2-5; DI; 1-5] ↓CuZnSOD [PI, MI, DI; 1-5] ↓MnSOD [PI, DI; 3-5; MI; 1-5] ↓CAT [PI; 2-5; MI, DI; 3-5] ↓GPx [PI, DI; 2-5; MI, DI; 1-5] ↓GST [PI, DI; 2-5; MI, DI; 3-5]	↓FBW[2-5] ↓SGR[2-5] ↓FI [1-5] ↓FE [2-5]	n/a	n/a	PI= 16.46[4]; 17.64[5] µg kg $^{-1}$ tissue MI= 15.90[3]; 18.54[4]; 20.34[5] µg kg^{-1} tissue DI= 18.91[3]; 24.40[4]; 28.82[5] µg kg $^{-1}$ tissue	● Initial weight: 12.17 ± 0.01 g; 60 days trial ● Malformations: missing of pelvic fin[2]; caudal fin deformity[3]; operculum and the curvature of vertebral column[5] ● DON caused the oxidative damage, apoptosis and the destruction of tight junctions via *Nrf2*, *JNK* and *MLCK* signaling in the intestine of fish, respectively. ● "the safe dose of DON for grass carp were all estimated to be 318 µg kg $^{-1}$ diet"

				↓GR [PI; 3-5; MI, DI; 2-5] ↓GSH [PI, MI; 2-5; DI, DI; 1-5]					
Rainbow trout *(Oncorhynchus mykiss)*	[#1]0; 1,166[1] and 2,745[2] µg kg [-1] [#2]0; 367[1] µg kg [-1]	Gonçalves *et al.* 2018	[#1]L[2]	n/a	[#1]↓FBW [2] [#1]↓SGR [2] [#1]↓SGR [2] [#1]↓FI [2] [#1]↓TGC [2] [#2] ↓FBW [Tf, 1]	[#1]↑ALT [2] [#1]↑AST [2] [#1]=LDH [#2]=LDH; ALT; AST; ALP, TP, Hct	N [#1]↓M [C; 1,2]	n/a	● [#1]Experiment 1: Initial weight: 14.10±0.05 g ; 50 days study ● [#1] Trout's challenged [C] by bath with *Yersinia ruckeri* ● [#2]Experiment 2: Initial weight: 89±8 g; 168 days study; sampling at day 37, 62, 92, 125 and 168[Tf]. ● [#1] Some trout's fed[2] showed abnormal body conformations and protruding anal papilla.
Rainbow trout *(Oncorhynchus mykiss)*	37.42[1]; 4,714[2]; 11,412[3] µg kg [-1]	Gonçalves *et al.* 2018	↓PR [T2, 2,3] ↓FR [T2, 3] ↓ER [T2, 3] ↓*igf1* [T2, 2,3] ↓*igf2* [T2, 2,3] ↑*npy* [T2, 2,3] ↑*adcyap1a* [T2, 2,3] ↑*try3* [T2, 2,3]	n/a	↓FBW [T1,T2, 2,3] ↓SGR [T1,T2, 2,3] ↑FCR [T1,T2, 3] ↓FI [T1,T2, 2,3] ↓PER [T1,2,3] ↓PER [T2,3]		N [T1] Y [T2, 3]	n/a	● Initial weight: 2.52 ± 0.03 g; 60 days study; sampling at 29[T1] and 60 [T2] days. ● Experimental diets were contaminated with ZEN (78.63[1-3]) and FB_1 (67.73[1-3]) besides added DON. ● No clinical signs (except anorexia at the higher DON dosage) were observed ● DON is metabolized to DON-3-sulfate
Rainbow trout *(Oncorhynchus mykiss)*	300[1]; 800[2]; 1,400[3]; 2,000[4]; 2,600[5] µg kg [-1]	Hooft *et al.* 2011	L[3-5]	n/a	↓FE; ↓FI; ↓TGC ↓WG; ↓RN ↓RE ↓NRE ↓ERE	n/a	N	n/a	● Initial weight: 24.3 g, 8 weeks study (samplings every 28 days)
Rainbow trout *(Oncorhynchus mykiss)*	(300[1]; 1,000[2]; 1,500[3] and 2,000[4] µg kg [-1]) x CFA	Hooft *et al.* 2017	n/a	n/a	↓FI ↓TGC ↓WG ↓RN ↓RE ↓NRE ↓ERE	n/a	N	n/a	● Initial weight: 1.8 g, 12 weeks study (samplings every 28 days) ● Tested an anti-mycotoxin feed additive (CFA) at 2g kg [-1] diet; ● No differences on feed efficiency
Rainbow trout *(Oncorhynchus mykiss)*	225[1] and 2,000[2] µg kg [-1]	Matejova *et al.* 2015;	n/a	K[2] ↑*TNF* ↑*IL-8* =IL-1β; IL-10 Sp[2] =*TNF; IL-8;* IL-	n/a	n/a	n/a	n/a	● 1-year old trout's, 23 days study ● Diet with 225[1] µg kg [-1] sever as control ● Levels of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, fumonisin B1 and B, HT-2

				1β; IL-10 = Lyz; IgM					toxin, T-2 toxin, nivalenol, ochratoxin A, and zearalenone were determined in the control and experimental diets ●The commercial diet (BioMar, Denmark) contained rapeseed oil, blood meal, fish meal, soya cake, sunflower cake, rapeseed meal, horse beans, wheat, soya concentrate, fish oil, pea protein, vitamins, and minerals
Rainbow trout *(Oncorhynchus mykiss)*	225[1]; 2,000[2] µg kg $^{-1}$	Matejova *et al.* 2014	K[2] N (G; Sk; L; Sp)	n/a	TL, SL, BW, LW CF,HSI	↓MCH[2] ↓Glu[2] ↓NH_3[2] ↓Cho[2]	n/a	n/a	● 1-year old trout's, 23 days study ● Diet with 225[1] µg kg $^{-1}$ sever as control ● Visual DON-haemorrhages in the liver and gastrointestinal tract ● No differences on RBC, Hb, Hct, MCV, MCHC, WBCs, Alb, TP, TAG, Lac, ALP,ALT. AST, LDH, Ca^{2+}, iP
Rainbow trout *(Oncorhynchus mykiss)*	<5, 410, 590 µg kg $^{-1}$	Ryerse *et al.* 2014	n/a	N	↓FI	n/a	N[C]	n/a	● Initial weight: 4.3 g, 7 weeks study ● Challenge[C] by intraperitoneal injection of *F. psychrophilum* (21 days infection) ● Restricted feed intake provided a protective effect for rainbow trout infected with *F. psychrophilum.*
Rainbow trout *(Oncorhynchus mykiss)*	0, 330[1] and 640[2] µg kg $^{-1}$	Ryerse *et al.* 2015)	n/a	n/a	↓FI	PCV, Lyph, Mono, Gran	↓M[2,C]	n/a	● Initial weight: 9.5 g, 7 weeks study ● Challenge[C] by intraperitoneal injection of *F. psychrophilum* (21 days infection) ● Restricted feed intake provided a protective effect for rainbow trout infected with *F. psychrophilum.*
Rainbow trout *(Oncorhynchus mykiss)*	0, 330[1] and 640[2] µg kg $^{-1}$	Ryerse *et al.* 2015) Exp II	n/a	n/a	↓FI ↓WG N FE	n/a	n/a	n/a	● Initial weight: 103.2 g, 21 days study ● Respiratory burst was significantly increased in trout fed diets containing DON ● The antimicrobial activity of DON was examined by subjecting *F. psychrophilum* in vitro to serial dilutions of the chemical. Complete inhibition occurred at a concentration of 75 mg L $^{-1}$ DON, but no effect was observed below this concentration (0–30 mg L $^{-1}$)

Rainbow trout *(Salmo gairdneri)*	0[0], 19,400[1]; 40,400[2]; 55,300[3]; 84,300[4]; 109,600[5] µg kg $^{-1}$	Woodward *et al*. 1983	n/a	n/a	↓FI[1-5;t1] ↓WG[1-5;t1] ↓FCR[1-5;t1]	n/a	N	n/a	● No fish weight available; fry stage; 8 weeks study ● Fish fed contaminated levels for 4[t1] weeks. After 4 weeks diets were changed to 1,940[0], 0[1], 4,040[2], 0[3], 8,430[4], 0[5], respectively, to test recovery. ● Natural contamination from mixture of ground husks, cobs and kernels, and contained approximately 4 µg zearalenone g $^{-1}$, a trace of 7-deoxyvomitoxin, but un-detectable levels of numerous other mycotoxins including nivalenol, 3-acetyldeoxynivalenol, fusarenone-X, diacetoxyscirpenol, T-2 toxin, neosolaniol and HT-2 toxin ● Feed refusal[1-5]; FI got normal when diets were changed
Rainbow trout *(Salmo gairdneri)*	0, 1,000[1], 2,000[2], 4,900[3], 7,500[4], 12,900[5] µg kg $^{-1}$	Woodward *et al.*, 1983) EXP II	n/a	n/a	↓FI[1-5] ↓WG[1-5] ↓FCR[1-5]	n/a	N	n/a	● Initial weight: 50 g, 4 weeks study ● Naturally contaminated diets
Zebra fish *(Danio rerio)*	<20, 118; 534; 1,543; 2,002; 3,022 µg kg $^{-1}$	Jørgensen 2012 and Sanden *et al*. 2012	N	n/a	=TL =CF =FW =SGR	=Lymph =Mono =Gran	n/a	n/a	● Initial weight: 9-12 cm in length, 45 days study ● No effects were detected at oxidative stress (CuZn sod, mapk 14, cyp1A), at cell cycle level (caspase 6, cyclin G1, PCNA), immune system (interleukin 6), appetite regulation (ghrelin) or at intestinal function level (maltase glucoamlyse, sodium glucose cotransporter).
Shrimp									
Black tiger shrimp *(Penaeus monodon)*	500[1]; 1,000[2] and 2,000[3] µg kg $^{-1}$	Supamattaya et al. 2005	↓FR[1-3]	N	↑Gr[1,2] ↓SGR[1-3]	↓ALP[1-3] ↓SGOT[2,3] ↓SGPT[2,3]	N	< 50,000 µg kg $^{-1}$ (LOD)	● Initial weight: 2 g; 8 weeks study ● No differences on THC or PO_aw and Ca^{2+} levels ● No differences in tissues: G, AG, HP, HT
Pacific white shrimp *(Litopenaeu*	0, 200[1], 500[2] and 1,000[3] µg kg $^{-1}$	Trigo-Stockli *et al*. 2000	n/a	n/a	↓W[3,t1-t4] ↓W[1,2,t4] ↓Gr[3,t1-t4] ↓W[1,2,t4]	n/a	N	N	● Initial weight: 1.7±0.05 g, 16 weeks study (samplings at 4[t1], 8[t2], 12[t3] and 16[t4] weeks) ● Naturally contaminated hard red winter wheat

s vannamei)					=FCR				

Revision of literature on the effects of DON multi-exposure

Species	Mycotoxin contamination (µg kg $^{-1}$)	Reference	Tissue alterations	Immunosuppressive	Growth performance alterations	Hematopoietic alterations	Increased mortality	Residues in tissues	OBS.
Common carp *(Cyprinus carpio)*	**AFB_1**=0[1], 0.01[2] and 0.02[3] µg L^{-1} **DON**= 0[4], 0.25[5], 0.5[6] µg L^{-1} **AFB_1:DON** = 0.01:0.25[7]; 0.01:0.5[8]; 0.02:0.25[9]; 0.02:0.5[10]	He et al. 2010	↑IR*[2,3,5,6] ↑↑IR*[7-10] ↑AST[2,3,5,6, 7-10, T1] ↑ALT[2,3,5,6, 7-10, T1-T3] ↑LDH[2,3,5,6, 7-10, T1]	n/a	n/a	n/a	n/a	n/a	● *Primary hepatocytes were used in this study ● Enzyme Activity of AST, ALT and LDH in Cell Supernatant sampled at 4[T1], 8 [T2] and 16h [T3]

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Fishes - Physiological parameters abbreviations: Alb= Albumin; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; ASA= anti-superoxide anion; AHR= anti-hydroxyl radical; ARE = Ash retention efficiency; CAT= Catalase; Cho= Cholestrol; CF= Condition factor; CuZnSOD= copper/zinc superoxide dismutase; Ery.N.L= Erythrocyte Nucleus length; Ery.N.W= Erythrocyte Nucleus width; EryL= Erythrocyte length; ERE= energy retention efficiency; FBW= Final body weight; FCR= Feed conversion ratio; FE= Feed Efficiency; FI= Feed intake; FW= Final weight; FTL= Final total length; Glu= Glucose; GR= glutathione reductase;GSH= Glutathione; GPx= Glutathione peroxidase; GST= Glutathione S transferase; GSH= Glutathione; Gran= Granulocytes; Hb= haemoglobin; Hct= haematocrit; HIS= hepatosomatic index; Lact= lactate; Lyz= Plasma lysozyme concentration; LDH= lactate dehydrogenase; Lyph= Lymphocyte; MDA= Malondialdehyde; MCH= mean erythrocyte haemoglobin; MnSOD= manganese superoxide dismutase; Mono= Monocytes; NRE= nitrogen retention efficiency; PC= protein carbonyl; PCV= Packed-cell volume; PER= Protein efficiency ratio; PRE= Crude protein retention efficiency; PR= Protein retention; PROD= Pentoxyresorufin O-depentylase; RE= recovered energy; ROS= Reactive oxygen species; RN= retained nitrogen; SL= Standard length; SGR= Specific growth rate; SOD= Superoxide dismutase; SSi= spleen somatic index; T-AOC= total antioxidant capacity; TGC= Thermal growth coefficient; TL= Total length; TP= Total protein; WBCs= Total leucocyte count (white blood cells); WG= Weight gain

Fishes - Tissues or tissue related abbreviations: DI= distal intestine; Ds= Dilation of sinusoids; FR= Fat retention; FA= Fat aggregation; G= gills; Hp= Hyperaemia; Ig= immunoglobulin; ILgth= intestine length (cm); ILI= intestine length index; ISI= intestinal somatic index; IW= intestine weight (g/fish); IHyp= intestinal hyperaemia; K = Kidney; L = Liver; Lp= Lipid peroxidation; LW= Liver weight; M = Muscle; MI= mid intestine; PI= proximal intestine; PR= Protein retention; SK = skin; Sp= Spleen; V= Vacuolization;

Fishes - Genes abbreviations: *Igf1*= insulin-like growth factor 1; *Igf2*= insulin-like growth factor 2; *npy*= neuropeptide Y precursor; *adcyap1a*= growth hormone-releasing hormone/pituitary adenylate or cyclase-activating polypeptide (PACAP); *try3*= trypsinogen 3 precursor; *TNF*= tumour necrosis factor; *IL* = interleukin; *GPx*=Glutathione peroxidase

Shrimps - Physiological parameters and tissues abbreviations: ALP= Alkaline phosphatase; CAT= Catalase; FCR=Feed conversion ratio; GPx.aw= Glutathione peroxidase activity; Gr=Growth rate; GSH= Glutathione; MDA= Malondialdehyde; mT-2= modified/masked mycotoxin (T-2); SGOT= Glutamic-oxaloacetic transaminase; SGPT= Glutamic-pyruvic transaminase; SGR=Specific growth rate; SOD= Superoxide dismutase; T-AOC= total antioxidant capacity; W= Weight; FR= Fat retention; HP=hepatopancreas; M= Muscle

1.3.3 Carry-over of mycotoxins

Bioaccumulation of mycotoxins from feed to animal food products might represent a direct risk to human health (CAST, 2003). Mycotoxin bioaccumulation in livestock is well investigated (Leeman *et al.*, 2007; Völkel *et al.*, 2011) and the risk to humans is evaluated by the European Food Safety Authority (EFSA) for several mycotoxins (AF's, OTA, ZEN, DON, FUM, T-2 and HT-2). Bioaccumulation of mycotoxins in poultry, swine and cows is managed by direct regulation of mycotoxins in animal feed (EC, 2002; 2006; EFSA, 2004a; b; c; d; 2005; 2011; 2013). While regulatory limits have been put in place for AF's (EFSA, 2004b), only guidance values are available for DON, OTA, FUM and ZEN (EC, 2006). This is because feed does not represent a direct risk for human health and because carry-over of these mycotoxins in terrestrial animals is expected to be low (EC, 2006), however, the same may not be true to the high diversity of aquaculture species.

Currently, no regulations or guidelines exist in order to avoid deposition of mycotoxins in farmed fish or shrimp, with the exception of fumonisins ($FB_1 + FB_2$ = 10,000 µg kg^{-1}; (EC, 2006)). Moreover, it is not taken into consideration that carry-over mechanisms in aquaculture farmed species might be different from terrestrial livestock species. Generally, the possibility of mycotoxin bio-accumulation/bio-magnification through the food chain due to the use of mycotoxin-contaminated non-plant origin ingredients such as animal by-products (e.g., shrimp head meal or chicken droppings; (Gonçalves *et al.*, 2020) or non-typical mycotoxin contaminated ingredients (e.g., fishmeal; (Gonçalves *et al.*, 2017)), is not taken into consideration and will not be addressed during this thesis, but may be consulted at Gonçalves *et al*. 2020.

Bioaccumulation of mycotoxins in aquaculture seafood products is not widely reported and consequently not regulated. This section will focus on documented peer-reviewed mycotoxin carry-over studies focussed in aquaculture species. Existing literature was reviewed, calculating transfer factors when the available data allowed, in order to compare bioaccumulation risks (Leeman *et al.*, 2007; Table S1.13). The transfer factor is expressed as the concentration of mycotoxin in animal tissues (µg kg^{-1}) divided by the concentration of the same mycotoxin in animal feed (µg kg^{-1}). This thesis will only focus on carry-over of DON, however, critical overview of existing literature for other mycotoxins (AF's, OTA, ZEN) on this topic may be consulted in the supplementary

material (AF's - S1.14; OTA – S1.15; ZEN/T-2 – S1.16) or in the original publication which this chapter is based on Gonçalves *et al.*, 2020.

1.3.3.1 Carry-over of deoxynivalenol

Deoxynivalenol bioaccumulation studies in aquaculture species are summarized in Table 1.3. In the case of the study reported by Nácher-Mestre *et al*. (2015) diets were co-contamination by FUM. This highlights the fact that co-occurrence of mycotoxins is extremely common (over 80 % of the cases (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d)).

In Atlantic salmon (*Salmo salar*), two studies are available ((Bernhoft *et al.*, 2017) and (Nácher-Mestre *et al.*, 2015)). Bernhoft *et al*. (2017) fed salmon with 2000 and 6000 µg kg^{-1} DON over the course of eight weeks and sampled liver, muscle, kidney and skin at three, six and eight weeks. The authors observed that both exposure dosages (2000 and 6000 µg kg^{-1} DON) led to DON deposition in the liver and muscle at all sampling points, except for the higher dosage at the last sampling point (eight weeks), at which DON was found in all sampled tissues (Table 1.3). In the case of the study performed by Nácher-Mestre *et al*. (2015), Atlantic salmon were fed lower levels of mycotoxins, however, with multi-occurrence. The three diets were formulated to have DON and FUM, but also minor levels of T-2 and 15-AcDON (Table 1.3). Salmon fed for six months with testing diets did not show detectable levels of DON and FUM in the sampled tissues. The same authors (Nácher-Mestre *et al.*, 2015) also studied bioaccumulation of mycotoxin co-occurrence (DON, 15-AcDON and FUM) in Gilthead sea bream (*Sparus aurata*) at two levels for 8 months. The authors did not observe mycotoxin deposition in muscle samples.

In common carp (*Cyprinus carpio*), Pietsch *et al*. (2014) observed that after feeding fish with 352, 619 and 953 µg kg^{-1} DON for four weeks, minor deposition of DON was observed in the muscle (Table 1.3). Interestingly, after the four weeks of DON exposure, fish were fed a non-contaminated diet for a period of two weeks and DON levels in the muscle were re-analysed. At the lower DON intake level (352 µg kg^{-1}), DON level in the muscle was higher after the depuration period (1.4 µg kg^{-1}) when compared to the level found at the end of feeding trial (eight weeks; 0.6 µg kg^{-1} DON). At the medium DON intake level (619 µg kg^{-1}), after the recovery period, a level of 0.7

µg kg $^{-1}$ DON was still found in the muscle, and at the higher level, however, no DON was detected after the recovery period.

In shrimp, two studies are available (Supamattaya *et al.*, 2005; Trigo-Stockli *et al.*, 2000; Table 1.3), in which both reported that DON was not detected in the muscle. Supamattaya *et al*. (2005) drew its conclusion after feeding black tiger shrimp black (*Penaeus monodon*) with 500, 1,000 and 2,000 µg kg $^{-1}$ DON for eight weeks. Trigo-Stockli *et al*. (2000) conducted its study using Pacific white shrimp (*Litopenaeus vannamei*), fed with 200, 500 and 1,000 µg kg $^{-1}$ DON for 16 weeks.

1.3.3.2 Additional remarks on the occurrence of mycotoxins in aquafeeds

DON occurrence in aquafeeds has been well documented in recent years (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Greco *et al.*, 2015; Nácher-Mestre *et al.*, 2015; Pietsch *et al.*, 2013). These mycotoxins have been pointed out as the main mycotoxin contaminants in aquaculture feeds, which is a reflection of the increasing inclusion levels of plant meals in diets, as these mycotoxins are produced in field conditions. However, DON bioaccumulation has been poorly studied in aquaculture-farmed species. In Atlantic salmon, two interesting and complementary studies are available (Bernhoft *et al.*, 2017) and (Nácher-Mestre *et al.*, 2015). While Bernhoft *et al*. (2017) proved the possibility of DON deposition in the liver and muscle in a relatively short exposure period (three weeks) with high DON levels (2,000 and 6,000 µg kg $^{-1}$ DON), Nácher-Mestre *et al*. (2015) showed no carry-over effects of FUM and DON co-contamination at low levels during long exposure periods. DON and FUM frequently occur together in aquaculture feed as both mycotoxins are produced by the same fungi species. Therefore, studies testing the effect of co-occurrence are particularly relevant. The levels tested were within the occurrence values reported in European aquafeeds (Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017), however, occasional high occurrences of DON and/or FUM should not be ignored (e.g., FUM occurrence reported by Gonçalves *et al*., 2017), as shown previously, levels up to 2,000 µg kg $^{-1}$ can lead to DON deposition in the muscle.

Contrary to Atlantic salmon, in common carp (*Cyprinus carpio*), Pietsch *et al*. (2014) showed that levels as low as 352 µg kg $^{-1}$ DON can lead to a minor deposition of DON in the muscle (Table 1.3). The author described that total DON elimination from the

muscle is a relatively long process, taking more than two weeks after stopping DON intake. Information about the complete elimination of DON is very important, as a fasting period before harvesting may be used to guarantee that DON or any other mycotoxin is eliminated during this period. However, in the study reported by Pietsch *et al*. (2014), the elimination period of DON in carp may be longer than the fasting period, which is normally 24 to 48 hours before harvesting. The study by Pietsch *et al*. (2014) highlighted that mycotoxin absorption, distribution, metabolism, and excretion (ADME) is entirely dependent on species, and data or conclusion extrapolations between species should be avoided. *Fusarium* mycotoxins (e.g., DON and FUM) are frequently present in plant commodities used for general aquaculture species, and taking into account the possible ADME differences depending on species and even on development stages, it would be very important to better understand the potential carry-over in the most important aquaculture species, giving a special emphasis to mycotoxin co-occurrence.

Despite the low number of studies on DON and FUM carry-over, apparently, its deposition in tissues seems to be very limited. However, its occurrence is frequent and due to its apparently long elimination period (generally higher than fasting period before slaughter, for the study species), its carry-over risk in aquaculture-farmed species should be better evaluated. Comparing transfer factors (TFs) obtained from Atlantic salmon and common carp, it seems that they are in line with the TFs of eggs, whole milk or meat (Table S1.13, Leeman *et al.*, 2007).

It is also important to highlight that the species investigated so far are cold/temperate water species. It is essential to increase the knowledge of the possible carry-over of *Fusarium spp*. mycotoxins in tropical species. Especially high-value species, normally exported, such as Pacific white leg shrimp, whose feeds have been identified recently as being contaminated with considerably high levels of DON (Gonçalves *et al.*, 2018b). Furthermore, these tropical species present a faster metabolism and consequently shorter fasting period before harvest is needed, which might greatly influence the deposition of mycotoxins in tissues.

Table 1.3: Documented deoxynivalenol and/or fumonisin carry-over in aquaculture species.

Reference	Species	Tested dosage	Mycotoxin detection level (µg kg $^{-1}$)	Transfer factor	Method of analysis	Observations
Fish studies						
Bernhoft *et al.* 2017	**Atlantic salmon** (*Salmo salar*)	0; 2,000[1] and 6,000[2] µg kg $^{-1}$ DON	L/M/K/SK (µg kg $^{-1}$) [1,t1] = 12.2/5.6/n.s./n.s. [1,t2] = 12.8/8.5/n.s./n.s. [1,t3] = 18.1/6.0/12.3/n.s. [2,t1] = 9.6/10.3/n.s./n.s. [2,t2] = 20.2/17.3/n.s./n.s. [2,t3] = 28.6/18.6/16.8/20.8	L/M/K/SK [1,t1] = 0.0061/0.0028/n.s./n.s. [1,t2] = 0.0064/0.0042/n.s./n.s. [1,t3] = 0.0091/0.003/0.0061/n.s. [2,t1] = 0.0016/0.0017/n.s./n.s. [2,t2] = 0.0034/0.0029/n.s./n.s. [2,t3] = 0.0048/0.0031/0.0028/0.0035	HPLC	▪ Initial weight: 58 g, 8 week study; ▪ Sampling at 3[t1], 6[t2] and 8[t3] weeks
Nácher-Mestre *et al.* 2015	**Atlantic salmon** (*Salmo salar*)	Diet 1 = 22.4 DON + 148 FUM Diet 2 = 19.4 DON + 754 FUM Diet 3 = 23.1 DON + 112 FUM	Not detected	n/a	LC–ESI–MS/MS	▪ 6 month trial ▪ Initial body weight of 228±5 g ▪ Minor amounts of T-2 found and 15-AcDON and OTA detected
Pietsch *et al.* 2014	**Common carp** *(Cyprinus carpio)*	352[1], 619[2] and 953[3] µg kg $^{-1}$ DON	Muscle samples (µg kg $^{-1}$) [1] = 0.6; [1, RP] = 1.4 [2] = 1.3; [2, RP] = 0.7 [3] = 1.2; [3, RP] = 0.0	Muscle samples [1] = 0.0017; [1, RP] = 0.0040 [2] = 0.0021; [1, RP] = 0.0011 [3] = 0.0013; [1, RP] = 0	HPLC	▪ Raised from eggs (average initial weight 36 g), 4 week study ▪ Additional 2 weeks of feeding uncontaminated diet – recovery period[RP]
Nácher-Mestre *et al.* 2015	**Gilthead sea bream** (Sparus aurata)	Diet 1 = 79.2 DON + 8.1 15-AcDON Diet 2 = 53.5 DON + 13.6 15-AcDON +6.4 FUM	Not detected	n/a	LC–ESI–MS/MS	▪ 8 month trial ▪ Initial body weight of 15 g up to 296 – 320 g

Huang et al. 2018	**Grass carp** *(Ctenopharyngodon idella)*	27; 318[1]; 636[2]; 922[3]; 1,243[4] and 1,515[5] µg kg $^{-1}$ DON	PI= 16.46[4]; 17.64[5] µg kg $^{-1}$ tissue MI= 15.90[3]; 18.54[4]; 20.34[5] µg kg $^{-1}$ tissue DI= 18.91[3]; 24.40[4]; 28.82[5] µg kg $^{-1}$ tissue	PI= 0.013[4]; 0.012[5] MI= 0.017[3]; 0.015[4]; 0.013[5] DI= 0.021[3]; 0.020[4]; 0.019[5]	HPLC	● Initial weight: 12.17 ± 0.01 g; 60 days trial ● Malformations: missing of pelvic fin[2]; caudal fin deformity[3]; operculum ● "the safe dose of DON for grass carp were all estimated to be 318 µg kg $^{-1}$ diet"; Huang *et al*. 2018
Shrimp studies						
Supamattaya *et al.* 2005	**Black tiger shrimp black** (*Penaeus monodon Fabricius)*	500; 1,000 and 2,000 µg kg $^{-1}$ DON	Not detected	n/a	HPLC	▪ Initial weight: 2 g; 8 week study ▪ No differences on THC or Ca^{2+} levels ▪ No differences in tissues: G, AG, HP, HT, * LOD given in the manuscript (50,000 µg kg $^{-1}$) seems to be very high; there is a chance of an error in the units
Trigo-Stockli *et al.* 2000	**Pacific white shrimp** (*Litopenaeus vannamei)*	0, 200, 500 and 1,000 µg kg $^{-1}$ DON	Not detected	n/a	HPLC	▪ Initial weight: 1.7±0.05 g, 16 week study (sampling at 4, 8, 12 and 16 weeks) ▪ Naturally contaminated hard red winter wheat

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC = High-performance liquid chromatography; LC–ESI–MS/MS = liquid chromatography-electrospray ionization-tandem mass spectrometry; TSQ= Quantum Access tandem mass spectrometer n/a = not applicable; n.s. not sampled

Tissue abbreviations: M = Muscle; L = Liver; K = Kidney; SK = skin.

1.3.4 Worldwide regulation of mycotoxins levels in aquafeed

The development of legislation is crucial to limit the dietary exposure of animals to mycotoxins as well to reduce the possibility of mycotoxin carry-over for edible animal tissues (previously discussed in chapter 1.3.3). However, as the awareness of mycotoxin-related issues in the aquaculture industry has only recently increased, specific recommendations for mycotoxin maximum levels in aquaculture feed do not yet exist, except for FUM, which are covered by guidance levels in the EU and the USA (Table S1.17). Contrary, in livestock species, mycotoxin levels in the feed are strictly regulated in several countries (Table S1.18). However, the maximum acceptable limits vary greatly from country to country (consult FAO, 2004). The European Union (EU) harmonized maximum levels and guidance values for mycotoxins in feed among its member states and for this sub-chapter only EU will be mentioned as the most regulated region (Table S1.18). In the EU, aquaculture feeds are only covered by maximum and guidance levels generally applicable to feedstuffs, which in many cases are higher than levels set for specific livestock species (compare Table S1.17 with Table S1.18). The only guidance value specifically addressing aquaculture species, namely fish is fumonisins ($FB_1 + FB_2$ = 10,000 µg kg^{-1}; (EC, 2006)).

1.4 Rainbow trout as a model species

Rainbow trout, *Oncorhynchus mykiss*; hereafter referred to as trout, is one of the most produced aquaculture species in the world. FAO (FAO, 2018) reported 814 thousand tonnes produced in 2016, representing 2% of the total aquaculture production in the world, which places this species within the top 15 of world most produced species. However, what makes trout a very interesting model in the context of this thesis is that trout is a predatory fish, placed at the highest trophic level of the aquatic environment food chain. Being a strict carnivorous species, trout has traditionally been fed high levels of fishmeal. Commercial feed formulations for salmonids, until recently, were incorporating 30 to 45% fishmeal (New and Wijkstroem 2002). For the sustainable growth of aquaculture sector, it is imperative to continually develop new aquafeeds in order to replace dietary fishmeal and fish oil [originating from threatened marine stocks] by using either plant ingredients or other ingredients. Salmonids are actually leading the fishmeal free diets revolution and the inclusion levels of fishmeal in salmon and trout have decreased over the years to levels of zero or close to zero inclusion. The successful replacement of fishmeal by other alternative protein sources, in salmonids, was

supported by a vast knowledge of nutrient requirements for these species as well as knowledge of the nutritional biochemistry and impact of anti-nutritional factors in the growth of these species (Alami-Durante *et al.*, 2010; Gomes *et al.*, 1993; Penn *et al.*, 2011; Pereira *et al.*, 2002). Contrary to marine species, also highly carnivorous and candidates for high levels of fishmeal replacement, the nutrition of salmonids in general [Atlantic salmon, Salmo salar; rainbow trout] and the impact of plant proteins on their performance is much better-documented than for marine species (Hardy *et al.*, 2002; Hardy, 1999). ANF's negative impact in salmonids performance and health, i.e., effects in binding nutrients, altering metabolic processes, decreasing intestinal enzyme activity, causing intestinal damage and reducing nutrient absorption are well documented (Francis *et al.*, 2002; Kraugerud *et al.*, 2007). Within ANF's, mycotoxins (detailed description in chapter 1.2) are probably the least study anti-nutritional factor, in aquaculture. It is curious, because mycotoxins and their effect in aquaculture species, namely trout, has been recognized threat since 1960. Where in this case an aflatoxin-contaminated cottonseed meal caused an outbreak of aflatoxicosis in hatchery-reared rainbow trout (*Onchorhynchus mykiss*) (Wolf and Jackson, 1963). The well-documented replacement of fishmeal by plant meals in salmonids, namely the impact of ANF's on its performance and health, makes trout an excellent model to study the impact of DON in this species.

1.5 Objectives and structure of the PhD research

This thesis has several objectives which may be summarized to a general goal of increasing the awareness of mycotoxin contamination in aquafeeds and their consequences to aquaculture species, especially characterizing the impact of deoxynivalenol in rainbow trout (*Onchorhynchus mykiss*).

Chapter 1 critically reviews the mycotoxin occurrence and co-occurrence in aquaculture finished feeds, and to understand the risk of mycotoxin carry-over to aquaculture seafood products. In a second step, it aimed to correlate the extent of mycotoxin contamination in aquaculture feeds to its impact in aquaculture species. Additionally, this chapter aims to expose the scientific community, the regulatory authorities and the aquaculture industry, to increase awareness of the main challenges and myths that the industry faces in developing mycotoxin management strategies.

Chapter 2 explores the impact of deoxynivalenol in rainbow trout and the difficulties to diagnose DON ingestion. It aimed to explore two different DON contamination scenarios, i.e., the effect of short-term feeding of high levels of DON and the effects of long-term feeding of low levels of DON. Moreover, we aimed to investigate the manifestation of clinical signs due to the ingestion of DON by inspecting several organs and tissues normally affected by the consumption of DON.

Chapter 3 targeted to further evaluate and elucidate the impact of DON on rainbow trout and study the reasons behind the apparent lack and/or high variability of clinical signs during DON ingested. This was attempted by characterizing the reduced growth performance by exploring the impact of DON on ingredient digestibility (total pepsin, trypsin, chymotrypsin, amylase and lipase activities) and its influence on the expression level of gene markers related to stress regulation, growth control, digestion regulation and appetite control. Moreover, due to the theoretical DON-related damage of the GIT, faeces was analysed for DON metabolites in order to study the DON metabolism.

Chapter 4 finalizes this thesis by further exploring the current knowledge of DON toxicokinetics and rainbow trout DON metabolism, accessing the organs assimilation rates, excretion and possible biotransformation kinetics.

CHAPTER 2

Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

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Abstract

The trend towards using plant-based ingredients in aquafeeds is set to intensify; however, mycotoxin contamination might be a challenge. Two diets, with deoxynivalenol (DON) levels of 1,166 µg kg $^{-1}$ (1.1 DON) and 2,745 µg kg $^{-1}$ (2.7 DON), were prepared for short-term DON-exposure (50 days). A third diet with a low DON level of 367 µg kg $^{-1}$ (0.3 DON) was prepared for long-term DON-exposure (168 days). Ingestion of DON by trout during both short-term/high-dosage exposure (50 days; 1,166 µg kg $^{-1}$ and 2,700 µg kg $^{-1}$ DON) and long-term/low-dosage exposure (168 days; 367 µg kg $^{-1}$ DON) impacted growth performance and, to a lesser extent, liver enzyme parameters (2.7 DON). Histopathology showed mild to moderate changes in the liver but not in the other sampled tissues (intestine and kidney). Despite these effects, short-term exposure of rainbow trout to high doses of DON did not result in increased susceptibility to *Yersinia ruckeri*. In both the short- and long-term studies, the effects of DON showed a high inter-individual variability. The present study confirms that sub-clinical levels of mycotoxins affect rainbow trout. The effects of such low mycotoxin levels could be masked by other production challenges while still negatively affecting productivity.

Keywords: Mycotoxins, *Oncorhynchus mykiss*, pathogen susceptibility, hepatocytes hyalinization

2.1 Introduction

In aquaculture, the trend to replace expensive animal-derived proteins, such as fishmeal, with more economical and sustainable plant protein sources has increased the probability of mycotoxin contamination in aquaculture feeds. According to Tacon *et al*. (2011), plant-based ingredients already represent the major dietary protein source used in feeds for lower trophic level fish species, such as tilapia, carp and catfish. These ingredients also account for the second major source of dietary protein and lipids after fishmeal and fish oil in the feed of shrimp and high trophic level fish species. Various plant sources have been used for salmonids but at lower inclusion levels than feed destined for lower trophic species. In most aquaculture species, plant protein choice and selection are based on a combination of local market availability, cost and the nutritional profile (including anti-nutrient content and level) of the plant meal in question (Davis and Sookying, 2009; Gatlin *et al.*, 2007; Krogdahl *et al.*, 2010).

The mycotoxin contamination of finished feeds and raw materials used in aquaculture as well as the negative effects of mycotoxins on aquatic species, particularly rainbow trout (*Oncorhynchus mykiss*), has been highlighted in recent publications (Gonçalves *et al.*, 2018e; Hooft and Bureau, 2017; Hooft *et al.*, 2011; Ryerse *et al.*, 2015; Tola *et al.*, 2015). However, mycotoxin contamination is not generally assessed in commercial aquafeeds or plant meals used to manufacture these feeds. Consequently, we do not have accurate estimates of the mycotoxin contamination levels in these commodities.

Few studies are currently available concerning mycotoxin occurrence in aquaculture plant meals and finished feeds. Gonçalves *et al*. (2018d) reported that deoxynivalenol (DON) was present in 68% of analyzed samples (shrimp and fish, sampled in Asia and Europe in 2014) at average contamination levels of 162 µg kg $^{-1}$ and maximum levels of 413 µg kg $^{-1}$. More recently, Gonçalves *et al*. (2018b) observed that contamination patterns for shrimp and fish feeds were slightly different, which likely reflects the type of commodity used for the different species. The authors observed that shrimp feeds were generally contaminated with low levels of DON, with the exception of some diets (contamination ranging from 329 µg kg $^{-1}$ to 2,287 µg kg $^{-1}$ of DON). In the case of fish feeds, samples were contaminated mainly by DON, up to a maximum level of 396 µg kg $^{-1}$, and were co-contaminated with other mycotoxins.

Trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with the initiation, elongation, and termination stages of this process (Kumar *et al.*, 2013). Knowledge of the effects of DON on aquatic species has increased recently (Gonçalves *et al.*, 2018e; Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2015; Ryerse *et al.*, 2015; Tola *et al.*, 2015), and studies on rainbow trout suggest that DON has a detrimental effect on feed intake, weight gain and feed efficiency (Hooft *et al.*, 2011; Ryerse *et al.*, 2015). Curiously, no effect has been detected on the immune status of animals fed with DON (Matejova *et al.*, 2014; Matejova *et al.*, 2015; Ryerse *et al.*, 2015).

In general, the effects of mycotoxicoses vary greatly depending on a variety of factors, including nutritional and health status prior to exposure, dose and duration of exposure, age, species and infection route. In addition, the lack of reliable clinical signs or parameters (including biomarkers) to correctly diagnose the ingestion of DON by aquatic species makes mycotoxin risk management in aquaculture very challenging.

The aim of the present study was to evaluate the effect of DON on rainbow trout under two different scenarios: first, the effect of short-term feeding of high levels of DON (50 days; 1,166 µg kg $^{-1}$ DON and 2,745 µg kg $^{-1}$ DON), and second, the effects of long-term feeding of low levels of DON (168 days; 367 µg kg $^{-1}$ DON). Moreover, we aimed to investigate the manifestation of clinical signs due to the ingestion of DON by inspecting several organs and tissues normally affected by the consumption of mycotoxins.

2.2 Materials and methods

2.2.1 Experimental diets

The experimental diets were formulated to be isoenergetic (22.20 kJ g $^{-1}$ dry matter (DM), isoproteic (52.20% DM) and isolipidic (17.90% DM) (Table 2.1). All diets were formulated with the same ingredients. Marine-derived ingredients (fishmeal and fish oil) represented 22.45% DM of the diet, whereas plant raw materials represented 59.70% DM of the diet. All ingredients were finely ground (hammer mill, 0.8-mm sieve), mixed, and then extruded (twin screw extruder, 2.0-mm pellet size, SPAROS, Portugal). The ingredients used to formulate the diets were subjected to liquid chromatography-tandem mass spectrometry, HPLC-MS/MS-based multi-mycotoxin analysis (University of Natural Resources and Life Sciences, Center for Analytical Chemistry Department

IFA, Austria), as described by Streit *et al*. (2013). The method covered major type A and B trichothecenes, zearalenone, fumonisins, aflatoxins and ochratoxins. For the purpose of data analysis, non-detect levels were based on the limits of detection (LOD) of the method used for analysis. The detected concentrations of major mycotoxins and of a selection of other fungal metabolites are listed in Table 2.2.

Table 2.1: Experimental control diet ingredients and proximate composition.

Ingredients	**Control (%)**
Fishmeal 60[a]	14.00
Fishmeal Super Prime[b]	12.45
Soy protein concentrate[c]	15.00
Wheat gluten[d]	12.30
Corn gluten meal[e]	8.00
Soybean meal[f]	6.00
Wheat meal[g]	6.40
Corn meal[h]	10.00
Fish oil[i]	10.00
Soy lecithin[j]	2.00
Antioxidant[k]	0.30
Monocalcium phosphate[l]	1.50
L-lysine[m]	0.50
DL-methionine[n]	0.50
Vitamin E[o]	0.05
Vitamin and mineral premix[p]	1.00
Proximate composition (% dry matter [DM])	
Dry matter	91.7 ± 0.0
Crude protein	52.2 ± 0.1
Crude fat	17.9 ± 0.0
Ash	9.3 ± 0.0
Gross energy, kJ/g DM	22.2 ± 0.0

[a] COFACO 60: 62.3% crude protein (CP), 8.4% crude fat (CF), COFACO, Portugal; [b] Super Prime: 67.4% CP, 8.2% CF, EXALMAR, Peru; [c] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [d] VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France; [e] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [f] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; [g] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal; [h] Corn meal: 8.1% CP; 3.7% CF, Casa Lanchinha, Portugal; [i] SAVINOR, Portugal; [j] Lecico P700IPM, LECICO GmbH, Germany; [k] Paramega PX, Kemin Europe NV, Belgium; [l] MCP: 22% P, 18% Ca, Fosfitalia, Italy; [m] Lysine HCl 99%, Ajinomoto Eurolysine SAS, France; [n] DL-Methionine 99%, EVONIK DEGUSSA GmbH, Germany; [o] ROVIMIX E50, DSM Nutritional Products, Switzerland; [p] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

Table 2.2: Multi-mycotoxin analysis of experimental diets

Analyte	Concentration (µg kg $^{-1}$)	Analyte	Concentration (µg kg $^{-1}$)
Major mycotoxins		**Other *Fusarium* metabolites**	
Aflatoxin B1	<LOD	15-Hydroxyculmorin	48.33
Zearalenone	11.44	Culmorin	69.87
Deoxynivalenol (DON)	<LOD	Equisetin	10.39
Fumonisin B1	<LOD	Fusaric acid	65.56
Fumonisin B2	25.05	***Penicillium* metabolites**	
Fumonisin B4	16.11	Brevianamid F	194.30
Ochratoxin A	<LOD	Mycophenolic acid	88.91
Sum of Ergot alkaloids	0.72	Rugulusovin	244.20
		Other *Aspergillus* metabolites	
Zearalenone metabolites		Tryptophol	28.90
Zearalenone-sulfate	32.62	**Other metabolites**	
		Cyclo(L-Pro-L-Val)	1,631.00
		Cyclo(L-Pro-L-Tyr)	2,004.00

DON	**Target concentration**	**Analyzed concentration**
Control	0.0	0.0
1.1 DON	1,500	1,166 ± 140
2.7 DON	3,000	2,745 ± 330
0.3 DON	400	367 ± 66.80

Note. Limits of detection (LOD) for aflatoxin B1 = 0.3 µg kg $^{-1}$. For DON and ochratoxin A, detection limit are 10, 50, and 0.2 µg kg $^{-1}$ and for fumonisin B1 the detection limit are 25 µg kg $^{-1}$. Five samples per diet were analysed.

Diets with three different levels of DON were prepared by adding DON (Romer Labs Diagnostic GmbH, Austria) to the feed during diet ingredient mixing. Two diets, with DON levels of 1,166 µg kg $^{-1}$ (1.1 DON) and 2,745 µg kg $^{-1}$ (2.7 DON), were prepared for short-term DON exposure (50 days). A third diet with a low DON level of 367 µg kg $^{-1}$ (0.3 DON) was prepared for long-term DON exposure (168 days). All diets were dried at 45 °C for 12 hours after the addition of DON and were stored at 4 °C until use.

Contamination levels were chosen taking into account previous literature on the effect of DON on rainbow trout (Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Matejova *et al.*, 2015; Ryerse *et al.*, 2015) as well as the reported DON levels in worldwide finished feed samples (Barbosa *et al.*, 2013; Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Greco *et al.*, 2015). The long-term exposure to DON attempts to mimic the most recently reported levels of DON in finished feeds ((Gonçalves *et al.*, 2018b), average of 82.87 µg kg $^{-1}$ and maximum of

396 µg kg $^{-1}$). However, the authors are aware that reports of mycotoxin occurrence in European aquaculture finished feeds are still very limited, and levels reported may vary annually (e.g., average DON contamination of 160.86 µg kg $^{-1}$ in 2014, of 165.61 µg kg $^{-1}$ in 2015, and of 87.87 µg kg $^{-1}$ in 2016; (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Generally, Asian aquafeed samples present higher DON levels compared with European aquafeed samples.

2.2.2 Fish and experimental conditions

This study was approved by the institutional ethics committee and the national authority according to §26 of Law for Animal Experiments, Tierversuchsgesetz 2016—TVG 2012 under No. BMWFW-68.205/0143-WF/V/3b/2015 and BMWFW-68.205/0058-WF/V/3b/2016. Rainbow trout (*Oncorhynchus mykiss*) originating from a farm with no prior history of *Yersiniosis* was used in both experiments. On arrival, the kidneys of ten fish were sampled, and their infection-free status was confirmed by culture-based analysis and polymerase chain reaction (PCR)-based analysis using *Yersinia ruckeri* specific primers (del Cerro *et al.*, 2002).

2.2.3 Short-term exposure to DON

For the experiment with short-term exposure to DON, 180 fish (14.10 ± 0.05 g) were randomly allocated to three treatments in quadruplicate and given either standard feed (control, CTRL), feed contaminated with 1,166 µg kg $^{-1}$ DON (1.1 DON) or feed contaminated with 2,745 µg kg $^{-1}$ DON (2.7 DON). Each aquarium of 85 L was supplied by a flow-through system with a temperature of 15.47 ± 0.14 °C, oxygen concentration of 8.73 ± 0.12 mg L $^{-1}$, and pH of 7.53 ± 0.04, with 0.0 ± 0.0 mg L $^{-1}$ total ammonia nitrogen, nitrites and nitrates. The fish were hand-fed the prepared diets (CTRL, 1.1 DON or 2.7 DON) three times per day near satiety for 50 days prior to performing the *Y. ruckeri* challenge.

2.2.4 Long-term exposure to DON

For the long-term exposure experiment, 120 fish weighing 89 ± 8 g were randomly allocated and distributed among eight tanks, each with a volume of 1 m ^{3}, supplied by a flow-through system with a water temperature of 18.6 ± 1.0 C, oxygen concentration of 8.56 ± 0.26 mg L $^{-1}$ and pH of 7.35 ± 0.35. Each tank contained 15 fish that were fed restrictively (2.5% of the average body mass) with either control feed (CTRL, 4 tanks)

or the control feed supplemented with 367 µg kg $^{-1}$ DON (0.3 DON, 4 tanks) for 168 days. The same quantity of feed (2.5% of the average body mass) was distributed in each tank by hand feeding and was adjusted after intermediate weighing periods (at 37, 62, 92 and 125 days). Five fish per replicate tank were subjected to moderate anesthesia (tricaine methanesulfonate (MS222) (Sigma-Aldrich Co., LLC, Bellfonte, USA) at a dose of 0.7 g L $^{-1}$, and a blood sample was collected by puncture of the caudal vein with a heparinized syringe at the beginning of the trial and at 62 and 125 days. Part of the blood sample was used for the determination of hematocrit, which was determined for five fish per treatment. Blood was transferred into hematocrit capillary tubes (Hirschmann), the tubes were then centrifuged at 13,000 RPM for 5 minutes (Hettich Haematokrit 200), and the percentage of red blood cells to sera was measured. The remaining part of the blood sample was centrifuged at 1,590 x g for ten minutes, after which the plasma (i.e., the supernatant fraction) was transferred to Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80 C until subsequent analysis of total protein. Total protein was determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard. All measurements were performed in a Synergy HT multi-mode microplate reader (BIOTEK, Vermont, USA).

2.2.5 Growth performance

All fish, in both the short- and long-term exposure experiments, were weighed to determine the initial individual body weight at the start of the experiments. In the short-term exposure study, the fish were weighed individually at the end of the 50-day period, and their total length was measured and recorded. Feed intake was recorded daily. In the long-term exposure study, the fish were weighed individually after 37, 62, 92,125 and 168 days.

The following calculations were made in both experiments.

The thermal-unit growth coefficient (TGC) was expressed as the growth rate and was calculated for each aquarium as [100 × (FBW 1/3 – IBW 1/3) / Σ (Temp (°C) × number of days)], where FBW = final body weight (g fish $^{-1}$) and IBW = initial body weight (g fish $^{-1}$).

The feed conversion ratio (FCR) was calculated as crude feed intake/weight gain, where FI = total dry feed/number of fish.

The protein efficiency ratio (PER) was calculated as weight gain (g)/protein intake (g).

The specific growth rate (SGR) was calculated as [(ln final weight − ln initial weight)/time in days] × 100).

Fulton's condition factor, K, was also used to measure individual fish health: $K = 100(BW/L^3)$, where BW is the whole body wet weight (g) and L is the length (cm). A factor of 100 was used to transform K to approximate a value of one.

2.2.6 Liver enzymes

In the short-term/high DON exposure experiment, five fish from each aquarium were sampled at the end of the experiment (50 days) for analysis of liver enzymes in blood. In the long-term/low DON exposure study, five fish from each aquarium were sampled on day 62 and on day 125. The fish were anaesthetized by immersion in tricaine methanesulfonate (MS222) (Sigma-Aldrich Co., LLC, Bellfonte, USA) at a dose of 0.7 g L^{-1} prior to blood collection. Blood samples were analyzed to measure the activities of lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate aminotransferase (AST) using a Spotchem EZ SP-4430 reader and Spotchem II GPT/ALT, Spotchem II LDH and Spotchem II GOT/AST kits (all products from Arkay, Amstelveen, Netherlands).

2.2.7 Histological examination

For the short-term/high DON exposure study, organs were sampled from 10 fish prior to the *Y. ruckeri* challenge and at the time of termination. The intestine, spleen, liver and kidneys (head and trunk kidney) of the fish were removed and fixed in 10% buffered formalin for 48 to 72 hours. The samples were embedded overnight in paraffin using a HistoMaster (Formafix, Düsseldorf, Germany). Sections (3 - 4 µm thick) were cut from each paraffin block and were left to dry overnight at 37 °C before being stained with hematoxylin and eosin.

The slides were evaluated under a light microscope (Nikon Eclipse E400, Feasterville, Pennsylvania). The following were examined: intestine (number of mucous cells in mucosa), liver (hepatocyte vacuolation, hepatocyte hyalinization, single cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation), and kidney (number of pigmented macrophage centers). To evaluate the number of cells, three high-power fields (HPF) were counted per slide.

2.2.8 Bacterial preparation

As a pre-trial to the challenge test, five groups of ten fish each were challenged by immersion with *Y. ruckeri* isolate 7959/11 to determine the appropriate infectious dose. *Y. ruckeri* isolate A7959/11 is a clinical isolate that originated from an outbreak at an Austrian trout farm in 2011. This isolate was kept at -80 °C on beads until three days prior to the start of the experiment. It was then inoculated on a blood agar plate and incubated at 22 °C. After 48 hours, a single colony was inoculated into 7.5 ml of BHI broth and was incubated in a shaking incubator at 20 °C with rotation at 150 rpm. After 10 hours, the cultures were evaluated by eye, and 2.5 ml was sampled from one culture and used to inoculate a 1.5-L BHI broth. This broth was then incubated for approximately 12 hours at 20 °C with shaking at 150 rpm.

2.2.9 Infection trial in the short-term exposure study

After 50 days, each feeding group of the short-term/high DON exposure study was further divided into two groups: two of the aquaria were infected with *Y. ruckeri* while fish in the two other aquaria were mock-infected with un-inoculated broth. In total, 90 fish were infected and 90 were mock-infected. The infection procedure was adapted from that described for *Aeromonas salmonicida* (Menanteau-Ledouble *et al.*, 2017). Briefly, bacteria were grown overnight in 1.5 L of BHI broth and their concentration was determined by measuring the optical density at a wavelength of 600 nm (OD600) per ml. Water circulation in the aquarium was interrupted, and the water volume was lowered to 50 L. The bacterial culture (2 ml) was added to each of the aquaria, yielding a final concentration of 2x104 CFU mL^{-1}. The fish remained in the solution for two hours, after which the water was progressively returned to its normal level and the circulation was reopened. The fish were monitored at least twice daily. Mortalities were recorded, and dead and moribund fish were immediately removed from the tanks. Moribund fish were euthanatized by prolonged immersion in a solution of 1 g L^{-1} of MS-222, and the kidney of the fish was sampled for microbial re-isolation of the pathogen on an agar plate. The colonies growing on these plates were examined and confirmed to be *Y. ruckeri* based on their morphologies. Furthermore, one in five isolates was selected; its genomic DNA was isolated using a Qiagen DNeasy kit, and PCR was performed using *Y. ruckeri* specific primers (del Cerro *et al.*, 2002). The surviving fish overcame the infection 17 days post-infection, at which point the

challenge was terminated. All remaining fish were euthanatized by prolonged immersion in a solution of tricaine methanesulfonate (MS222; 1 g L^{-1} of water), weighed, measured and examined for gross clinical signs of enteric red mouth syndrome (oral congestion, hemorrhages or petechia, exophthalmia and ocular hemorrhages, ascites in the abdominal cavity, enlarged spleens and hemorrhages or petechia in the internal organs, bloody intestines or adipose tissues).

2.2.10 Clinical signs

During both experiments, gross clinical signs were assessed by visual examination of the fish at the time of termination. Lesions (hemorrhages and ulcerations) on the skin were recorded, as were any obvious abnormalities such as a protruding anal papilla. The state of the gills was recorded as well as the presence of anaemia, haemorrhages or necrosis.

The fish were examined internally for any abnormalities. In particular, record was made of congestions, petechia or haemorrhages of the internal organs. The colour of the liver and the size of the spleen were assessed, as was the general health of the intestine (in particular, the presence of congestion, haemorrhage or intussusception was determined).

2.2.11 Statistical analysis

All parameters such as the final weight, SGR, PER, FI, FCR, condition factor (CF), TGC, LDH, ALT and AST were subjected to analysis of variance in SPSS 21 for Windows (IBM Corp., Armonk, NY, USA). One-way ANOVA was performed, and differences between the means were tested by Tukey's multiple range test. The Shapiro-Wilk test was used to analyze the normality, and homogeneity of variances was tested using Levene's test. Data analyzed did not violate the assumption of equal variances and showed a normal distribution. All parameters expressed as percentages were subjected to arcsin square root transformation. Additionally, one-way ANOVA was performed to analyze the histological differences in the intestine (number of mucous cells in mucosa) and liver (single cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation) between the DON dietary treatments and controls.

Following the challenge, survival curves were constructed for each treatment, and Kaplan-Meier and odds ratio analyses were performed using SPSS v.20 (IBM) and MedCalc (Microsoft). The level of significance was set at $p < 0.05$, and the results are presented as the mean ± SD (standard deviation of the mean).

2.3 Results

2.3.1 Experimental diets

The four experimental diets were formulated to be isoenergetic (22.20 kJ g $^{-1}$ DM), isoproteic (52.20% DM) and isolipidic (17.90% DM) and to meet all the nutrient requirements for the species examined in the study. There was no significant difference ($p > 0.05$) between treatments regarding the nutritional composition of the experimental diets. Analysis of the feed to confirm mycotoxin levels showed DON contamination was successfully achieved, although observed levels were slightly lower than intended (Table 2.2). Other metabolites/toxins were found in the basal diet (common to all experimental groups) due to natural contamination of the plant raw materials used to formulate the diet (Table 2.2). Generally, these metabolites/toxins, produced mainly by *Fusarium* and Aspergillus, were at levels below 100 µg kg $^{-1}$. Regarding the *Penicillium* toxins, *brevianamide F* and *rugulusovin* were found at levels of 194 and 244 µg kg $^{-1}$, respectively. Fungal and bacterial metabolites were also detected in the experimental diets, namely, cyclo (L-Pro-L-Val) and cyclo (L-Pro-L-Tyr) at relatively high concentrations (1,631 and 2,004 µg kg $^{-1}$, respectively).

2.3.2 Growth performance

2.3.2.1 Short-term DON exposure

The results showed that rainbow trout was sensitive to the DON levels tested (Table 2.3).

Table 2.3: Growth performance parameters determined in the short term/high DON dosage study.

	Final Weight (g)	**SGR (% day $^{-1}$)**	**PER**	**FI (g fish $^{-1}$)**	**FCR**	**CF**	**TGC**
Control	101.36±19.81 ^{a}	2.52±0.07 ^{a}	2.17±0.05	81.21±4.71^{a}	0.98±0.07	1.42±0.12^{ab}	0.113±0.005^{a}
1.1 DON	95.37±19.20 ^{a}	2.46±0.06 ^{a}	2.01±0.13	81.65±3.78^{a}	1.03±0.07	1.46±0.13 ^{b}	0.109±0.004^{a}
2.7 DON	79.91±16.54 ^{b}	2.20±0.09 ^{b}	2.01±0.07	64.03±2.87^{b}	1.05±0.04	1.39±0.12 ^{a}	0.094±0.005^{b}
One -way ANOVA							
p value	<0.001	<0.001	0.096	<0.001	0.423	0.033	0.001

Note. Data are presented as mean ± SD. Values in the same column with different letters are significantly different ($p < .05$). CF = condition factor; FCR = feed conversion ratio; FI = feed intake; PER = protein efficiency rate; SGR = specific growth rate; TGC = thermal-unit growth coefficient.

The presence of 2,700 µg kg $^{-1}$ DON in the diet led to a significant decrease ($p < 0.001$) in FI. The same treatment (2.7 DON) also resulted in a significant decrease in the final

weight (79.91 ± 16.54 g; $p < 0.001$), SGR (2.20 ± 0.09% day $^{-1}$; $p < 0.001$), TGC (0.094 ± 0.005; $p < 0.001$) and CF (1.39 ± 0.12; $p < 0.033$) compared to the controls (final weight = 101.36 ± 19.8 g; SGR = 2.52 ± 0.07% day $^{-1}$; TGC = 0.113 ± 0.005 and CF = 1.42 ± 0.12). Observations of the feeding behaviour of the DON-fed groups confirmed that the fish initially accepted the feed, and a reduction in FI was progressively established. We, therefore, assumed that the lower FI in the DON-fed groups compared to the control group was probably not due to the unfavourable organoleptic properties of DON-contaminated feed.

2.3.2.2 Long-term DON exposure

In the long-term exposure study, the fish that received the contaminated diet also showed lower farming performances (FBW, FCR and SGR) compared to the control. These differences increased over time (Figure 2.1, 2.2 and 2.3) and after 168 days of exposure to 367 µg kg $^{-1}$ DON, fish that ingested DON presented a final weight of 487.40 g compared to 593.63 g in the control group ($p = 0.053$, Figure 2.1). However, these differences were never statistically significant.

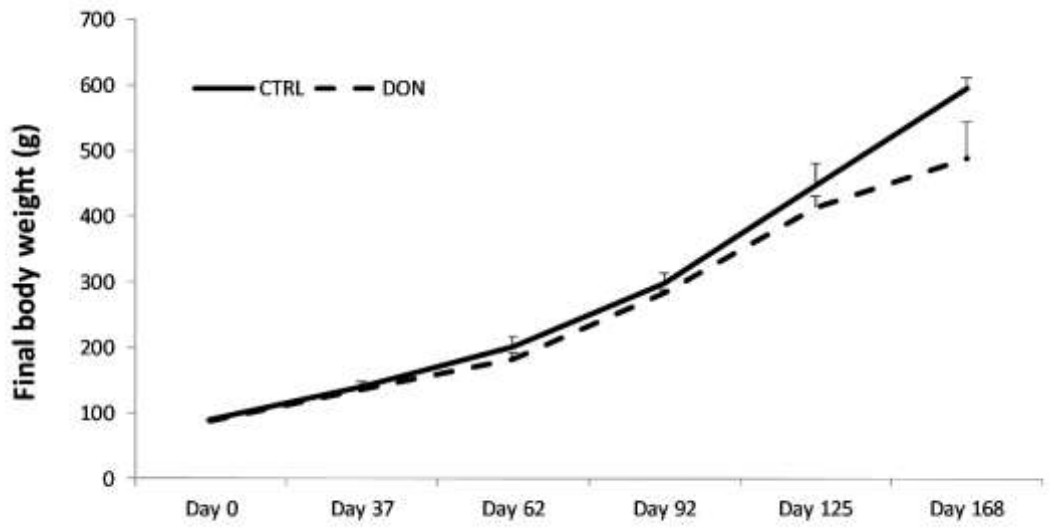

Figure 2.1: Growth curve representing the average weight of the fish during the long-term experiment.

A similar pattern of lower performance in the DON-fed animals was observed for FCR (Figure 2.2) and SGR (Figure 2.3): animals fed the control diet presented an FCR of 1.86 compared to 2.50 for DON-fed animals. PER was generally lower for animals that were fed DON and was significantly lower on day 92 ($p = 0.044$) and day 168 ($p =$

0.050; Table 2.4). Feed intake was generally higher for animals that were fed DON and was significantly higher on day 62 (p = 0.041; Table 2.5).

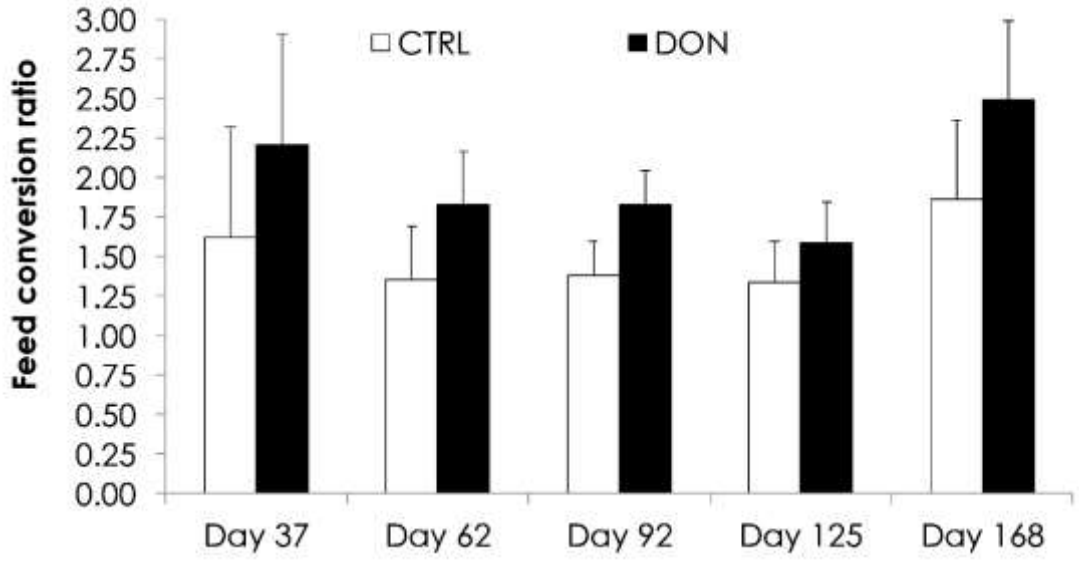

Figure 2.2: Feed conversion ratio at different sampling time points. Values are displayed as average ± SD.

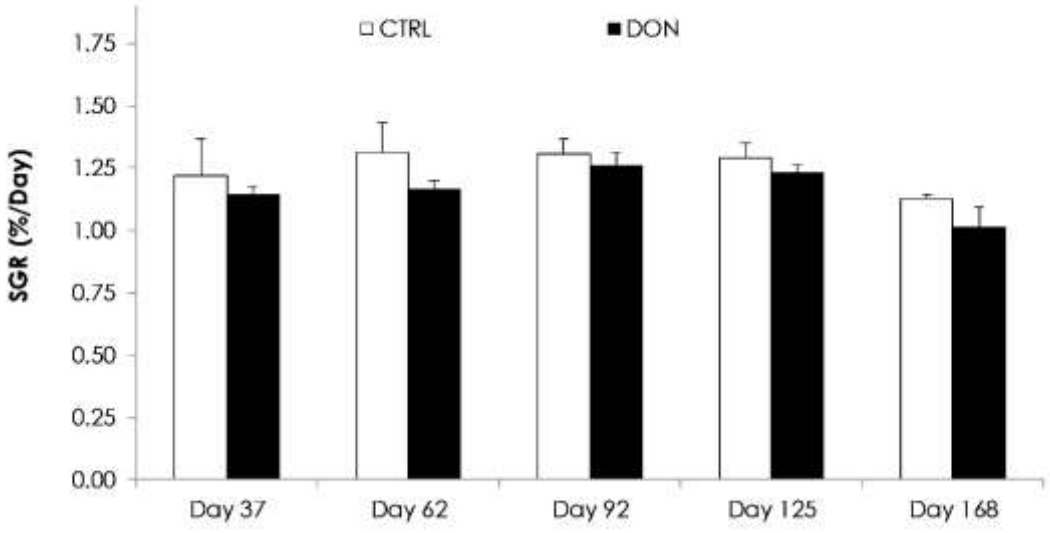

Figure 2.3: Specific growth rate at different sampling time points. Values are displayed as average ± SD.

Table 2.4: Protein efficiency rate at different sampling time points for the long-term/low deoxynivalenol (DON) dosage experiment

	Day 37	Day 62	Day 92	Day 125	Day 168
Control	1.15±0.17	1.38±0.18	1.34±0.11	1.38±0.13	0.99±0.03
0.3 DON	0.89±0.22	1.03±0.16	1.15±0.14	1.18±0.18	0.76±0.14
One-way ANOVA					
p value	0.150	0.044	0.110	0.183	0.50

Note. Values are displayed as mean ± SD

Table 2.5: Feed intake (% body weight/ day) at different sampling time points for the long-term/low deoxynivalenol (DON) dosage experiment

	Day 37	Day 62	Day 92	Day 125	Day 168
Control	1.92±0.06	1.67±0.08	1.61±0.07	1.43±0.09	1.41±0.03
0.3 DON	2.02±0.08	1.85±0.09	1.74±0.10	1.59±0.15	1.66±0.68
One -way ANOVA					
p value	0.133	0.041	0.109	0.189	0.070

Note. Values are displayed as mean ± SD

2.2.3 Histology

In the short-term exposure study, among the 2.7 DON groups, two out of ten animals showed mild to moderately hyalinized hepatocytes. In one trout, multiple areas of necrosis with scattered haemorrhages were present (Figure 2.4). Vacuolation of hepatocytes was also more pronounced in 2.7 DON animals (5 out of 10 fish) compared to the control animals (no registered cases of vacuolation of hepatocytes). In the 1.1 DON groups, hyalinized hepatocytes were visible (6 out of 10 fish), but to a lesser extent compared with the 2.7 DON groups (8 out of 10 fish). No significant differences were obvious between any of the experimental groups based on counts of the mucous cell numbers in the intestinal mucosa, pigmented macrophage centers in the liver and kidney, and number of necrotic single cells in the liver. No histological alterations were found in the intestine or kidneys (head and trunk kidney).

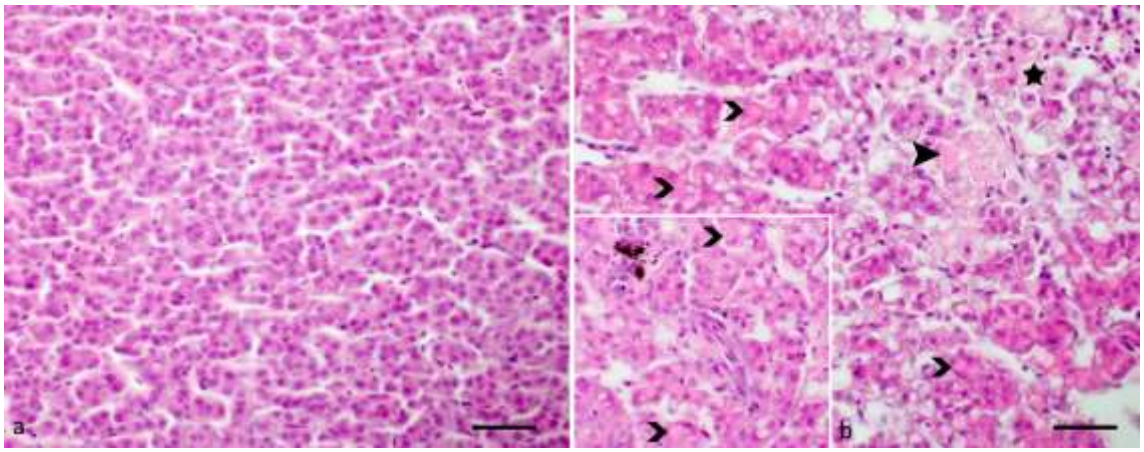

Figure 2.4: *Oncorhynchus mykiss*, histologic appearance of control (A) and 2.7 deoxynivalenol exposed fish (B); (A) normal structure of hepatocytes; (B) normal structure is disrupted, multiple hepatocytes are necrotic (star; observed in 1/10 fish sampled), scattered fibrin exudation (closed arrowhead; observed in 6/10 fish sampled), multiple hepatocytes show intracytoplasmatic eosinophilic, amorphous material (hyalinized hepatocytes) (open arrowheads; observed in 8/10 fish sampled), hematoxylin and eosin stain, bars = 50 μm; inlet: higher magnification showing hyalinized hepatocytes (open arrowheads)

2.3.4 Challenge test

Cumulative mortality after inoculation with *Y. ruckeri* is shown in Figure 2.5. The challenge trial lasted 17 days, and the 2.7 DON treatment showed a significantly higher survival rate ($p < 0.020$) compared to the control treatment. Controls exhibited 73.3% survival while the 1.1 DON and 2.7 DON treatments had a survival rate of 86.7% and 93.3%, respectively. No statistically significant differences were found between the 1.1 DON and 2.7 DON treatments or between the 1.1 DON treatment and the controls. The cause of death was confirmed as *Y. ruckeri* on the basis of the clinical signs. Furthermore, bacteria were re-isolated from the kidneys of infected fish. In each case, pure cultures were obtained, and the colonies displayed morphology consistent with *Y. ruckeri*. This was further confirmed by isolating the genomic DNA from selected colonies and performing PCR using the primers described by del Cerro *et al*. (2002). Fish that had recovered from the infection at the time of the challenge termination did not display any gross clinical signs. Similarly, non-infected fish did not display any signs of infection.

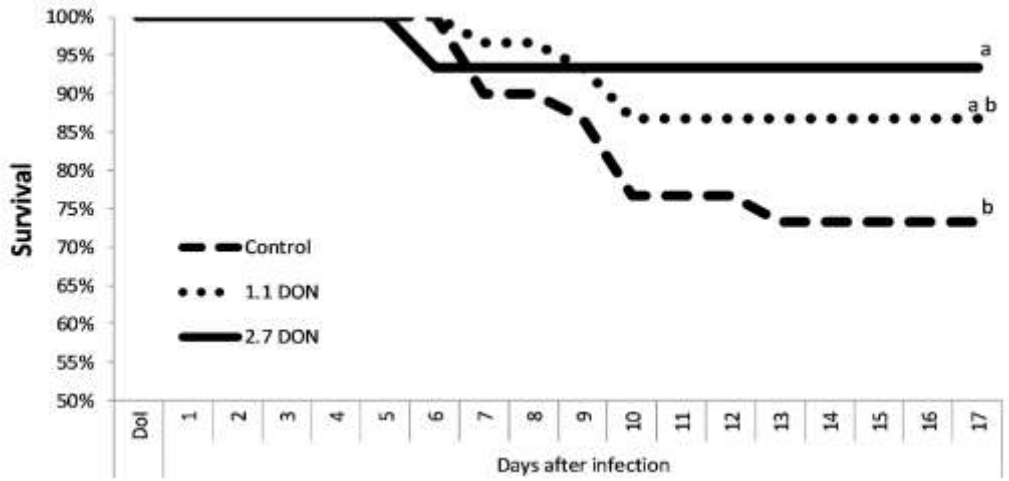

Figure 2.5: Survival curve following infection with *Yersinia ruckeri* during the high-dose experiment.

2.3.5 Liver Enzymes

2.3.5.1 Short-term DON exposure

The effects of the dietary treatments on LDH, ALT and AST activities in the serum are summarized in Table 2.6. Samples from the fish that received the dietary DON appeared to have a higher LDH activity, although these results were not statistically significant (p = 0.078). The 2.7 DON treatment showed a significant increase in ALT and AST activities (76.10 ± 9.88 IU L^{-1}; $p < 0.001$ and 876.50 ± 87.60 IU L^{-1}; $p < 0.001$, respectively) compared with the control (ALT = 14.20 ± 7.66 IU L^{-1} and AST = 389.70 ± 2.36 IU L^{-1}; Table 2.6).

Table 2.6: Effects of dietary treatments on lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate aminotransferase (AST) activities in the serum for short-term/high deoxynivalenol (DON) exposure experiment.

	LDH (IU L^{-1})	**ALT (IU L^{-1})**	**AST (IU L^{-1})**
Control	1000.60±187.01[a]	14.20±7.66[a]	389.70±2.36[a]
1.1 DON	2001.18±825.06[a]	22.00±0.97[a]	543.80±45.68[a]
2.7 DON	1700.60±163.27[a]	76.10±9.88[b]	876.50±87.60[b]
One -way ANOVA			
p value	0.078	<0.001	<0.001

Note. Data are presented as mean ± SD. Values in the same column with different letters are significantly different ($p < 0.05$).

2.3.5.2 Long-term DON exposure

Blood enzyme parameters measured at different sampling points are shown in Table 2.7. No significant differences were found during the experimental period for the different enzymes sampled.

Table 2.7: Effects of dietary treatments on lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (T-Prot), and hematocrit in the serum at different sampling time points for long-term/low deoxynivalenol exposure experiment.

	Sampling	**Hematocrit (%)**	**ALT (IU L $^{-1}$)**	**AST (IU L $^{-1}$)**	**LDH (IU L $^{-1}$)**	**ALP (IU L $^{-1}$)**	**T-Prot. (g L $^{-1}$)**
	Initial	51.2±0.08	17.2±11.8	432.9±157.2	1846.5±1178.2	*	*
Control	62days	39.9±3.32	11.1±3.5	309.1±239.6	1862.7±1199.4	143.4±71.8	3.0±0.67
Mycotoxins		37.6±4.29	24.4±25.4	385.2±91.55	2497.0±1573.1	171.6±69.5	3.0±0.5
Control	125days	*	*	324.7±144.4	1968.7±1222.8	154.4±47.72	3.4±0.79
Mycotoxins		*	*	216.5±97.3	914.8±314.9	146.3±69.11	3.1±0.63

Note. Asterisks denote values could not be determined due to technical problems with samples. Values are displayed as averages ± SD. n = 5 per treatment.

2.3.6 Clinical signs

Few clinical signs were observed in the fish exposed to the mycotoxin, and when abnormalities were observed, only a small number of fish were affected. Among the abnormalities were abnormal body conformations, observed in 15 out of 60 fish that were fed 2,745 ± 330 µg kg $^{-1}$ DON, characterized by a reduction in fish length in relation to width (Figure 2.6). In addition, in five out of 60 fish that were fed 2,745 ± 330 µg kg $^{-1}$ DON, a protruding anal papilla was observed (Figure 2.7). Intussusceptions were observed internally in two fish.

Figure 2.6: Abnormal body conformations, characterized by a fish length reduced in relation to its width. Observed in 15 fish out of 60 fish fed 2,745 ± 330 µg kg $^{-1}$ deoxynivalenol.

Figure 2.7: Fish presenting protruding anal papilla after being fed 2,745 ± 330 µg kg $^{-1}$ deoxynivalenol (DON). Observed in 5 fish out of 60 fish fed 2,745 ± 330 µg kg $^{-1}$ DON.

2.4 Discussion

The decreasing supply and rising cost of fishmeal have led the aquaculture industry to investigate alternative sources of protein to substitute fishmeal in aquafeeds. Plant-based meals seem to be one of the most promising solutions for replacing fishmeal, and numerous plant raw materials have been successfully tested (Gatlin *et al.*, 2007). However, recent studies have noted the occurrence of mycotoxins in plant-based aquafeeds (Barbosa *et al.*, 2013; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Greco *et al.*, 2015; Nácher-Mestre *et al.*, 2015; Pietsch *et al.*, 2013). In the present study, the experimental diets were contaminated with several mycotoxins and fungal metabolites in addition to the added DON. The presence of other mycotoxins and fungal metabolites highlights the risk of mycotoxin contamination in aquaculture finished feeds. The present experimental diet represents a typical commercial trout diet that contains plant-based compounds (59.70% DM). The co-occurrence of mycotoxins and fungal metabolites in this diet, even at low concentrations, may lead to synergistic/additive/antagonistic effects between these compounds, which cannot be ruled out as a contributing factor for the obtained results. However, further studies are needed to address possible interactions between mycotoxins, especially at low contamination levels.

The objective of the present trial was to evaluate the possible effects of DON contamination in aquaculture feeds under two different scenarios. In the first scenario, the effect of short-term feeding of high levels of DON (50 days; 1,166 µg kg $^{-1}$ DON and 2,745 µg kg $^{-1}$ DON) was examined in an attempt to mimic the potential inclusion of highly contaminated raw material(s) in the finished feed. This situation would normally only affect a few batches of feed; therefore, the exposure would occur over a short period. In this scenario, the potential influence of mycotoxins on *Y. ruckeri* susceptibility was also evaluated. The second experiment studied the effects of long-term exposure to low levels of DON (168 days; 367 µg kg $^{-1}$ DON). This experiment was designed to replicate a situation that is more commonly found because 367 µg kg $^{-1}$ DON is comparable to the average DON contamination level previously found in aquafeeds during recent years (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017).

One of the main constraints when researching mycotoxins in aquaculture species is the lack of mycotoxin-induced clinical symptoms. While it is true that several published

reports describe some clinical signs for the most common mycotoxins (see the review conducted by (Anater *et al.*, 2016)), most of these clinical signs are very general and can be attributed to any other pathology or challenge faced by the animals, e.g., anti-nutrition factors or lectins in the diet (Hart *et al.*, 2010). Furthermore, the clinical signs typically present high variability.

In the present manuscript, the occurrence of clinical signs was evaluated in both the short- and long-term exposure experiments, and special attention was paid to visual clinical signs. In the short-term/high DON exposure experiment, 15 out of 60 fish that were fed 2,745 ± 330 µg kg $^{-1}$ DON showed an abnormal body conformation, characterized by a fish length reduced in relation to its width (Figure 2.6), and five out of 60 fish from same treatment presented a protruding anal papilla (Figure 2.7). No clinical signs were observed after long-term exposure/low DON exposure. Although clinical manifestation was observed in a small number of individuals (only at the higher dosage of the short-term/high DON exposure experiment), it cannot be concluded that the signs observed are directly attributed to DON. The rectal prolapse observed in some fish is also described as a DON clinical manifestation in swine when fed 3,000 µg kg $^{-1}$ DON (Madson *et al.*, 2014). However, a recent study (Gonçalves *et al.*, 2018e) stated that no macroscopic lesions were found (i.e., internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) on rainbow trout that were fed high levels of DON (11,412 ± 1,141 µg kg $^{-1}$). Taking into account the previous study (Gonçalves *et al.*, 2018e) and three other studies with the same range of DON contamination (0.3 to 5.9 ppm), Hooft *et al*. (2011) and Ryerse *et al*. (2015) also reported no major pathological changes in the distal intestine of trout, while Matejova *et al*. (2014) found gastrointestinal haemorrhages. It is possible that the impact of DON might vary greatly depending on unknown factors, even for the same species.

Recently, Gonçalves *et al*. (2018e) reported a novel DON metabolite (DON-3-sulfate) found in rainbow trout faeces. The authors suggested that this biotransformation achieved by sulfation is probably realized by the trout gut microbiota as was previously described for other fish species (*Ameiurus nebulosus*; (Guan *et al.*, 2009)). This biotransformation, if achieved by the gut microbiota, can also help to explain the high individual variability obtained, as the capacity to metabolize DON will be directly influenced by the individual fish microbiome. This explains the absence of clinical signs in some of the fish that were fed DON because DON-3-sulfate is less toxic than DON.

The high inter-individual variation within the groups that were fed mycotoxins highlights the importance of the individual health and nutritional status prior to DON ingestion, as supported by other authors (Hendricks, 1994). Due to the reasons, previous stated, the clinical manifestation found in the present study, even if only present in a small number of individuals, should be further confirmed as a DON exclusive clinical sign, associating it with an individual fish microbiome.

Reduction in feed intake is a well-documented response of rainbow trout to diets contaminated with naturally occurring or artificially added DON (Gonçalves *et al.*, 2018e; Hooft *et al.*, 2011; Ryerse *et al.*, 2015). In the present short-term study, fish that were fed 2,745 µg kg^{-1} of DON showed a significant reduction ($p < 0.001$) in feed intake. However, no effect was observed in fish that were fed 1,166 µg kg^{-1} of DON. A significant decrease in growth was also detected in the 2.7 DON treatment; TGC decreased by 17% ($p = 0.001$), and SGR decreased by 13% ($p < 0.001$). However, no significant differences ($p > 0.05$) were found for PER or FCR. In the long-term study, ingestion of DON was asymptomatic, as the animals presented no clinical signs, and growth rate was slightly affected only after 92 days of ingesting DON. At the end of the trial (168 days), the animals that were fed DON weighed less than the control animals. While not significantly different, the tendency for reduced weight gain in animals that were fed DON is consistent with the short-term experiment. Recently, Gonçalves *et al*. (2018e) suggested that suppression of appetite due to DON contamination in feeds might be a defence mechanism to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The authors showed that PACAP (pituitary adenylate cyclase-activating polypeptide) seems to be fundamental for explaining the reduction of feed intake in DON-fed treatments, inducing anorexia, reinforcing the influence of DON on the hypothalamic melanocortin system. It is also important to mention that a contamination dose of 367 µg kg^{-1} of DON is a frequent and plausible level of contamination that is often found in aquafeeds incorporating plant meals (Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Moreover, this value is close to the limit of detection of most commercial ELISA (enzyme-linked immunosorbent assay) strip tests for DON, which means that samples need to be analyzed by more robust methods (e.g., HPLC), which increases costs and the time to receive sample results. The observed asymptomatic decrease in growth performance may lead to important economic consequences for the aquaculture industry.

In both experiments, it was difficult to correctly diagnose DON intake using the other parameters evaluated (liver enzymes and histology). In the short-term/high DON exposure study, histological and enzymatic changes showed different results, and individual variability was very high. Enzymatic activity was used to evaluate the possibility of tissue destruction. ALT and AST have previously been used as markers of liver dysfunction (Gül *et al.*, 2004; Saravanan *et al.*, 2012), and ALT is an intracellular enzyme that has been used as a marker of tissue destruction in the liver. However, no clear pattern could be observed in the studies. Only in the short-term/high-level DON exposure study were elevated ALT serum levels found in the 2.7 DON treatment compared with the control group. In addition, AST values were significantly higher in the 2.7 DON treatment compared with the control. Elevated ALT and AST serum levels might be an indication of liver or other parenchymal organ damage. Liver histopathology revealed mild to moderate damage in a limited number of DON-exposed fish. However, no histological alterations were detected in the intestine or kidneys (head and trunk kidney). DON is known to cause impairment of barrier integrity, affecting the lamina propria and tight junctions, which may increase GIT permeability and consequently allow the entry of luminal antigens and bacteria normally restricted to the GIT lumen (Dänicke *et al.*, 2004; Grenier and Applegate, 2013). The fact that histological alterations were not found in the intestines, despite the altered values of ALT and AST, might lead us to hypothesize that short exposure periods might not be sufficient to lead to histological alterations and/or that histology might not be a good method to evaluate negative DON effects in the intestines. Moreover, as mentioned by Gonçalves *et al*. (2018e), the individual microbiome seems to play an important role in DON biotransformation, which may also influence the obtained histological results. It would also be interesting to more closely examine the tight junction proteins as a more sensitive indicator for possible DON impact at the intestinal barrier, specifically at the tight junction level.

The results obtained for the *Y. ruckeri* challenge are consistent with the results from previous studies that investigated the effect of dietary DON on the mortality of rainbow trout challenged with other bacterial pathogens Hooft *et al*. (2011) and Ryerse *et al*. (2015). The apparent absence of an immunosuppressive effects on trout challenged with DON contrasts with published data for livestock species such as swine (Lessard *et al.*, 2015; Pierron *et al.*, 2016). An eventual direct suppression of *Y. ruckeri* by DON seems

unlikely as it is very well described that trichothecenes interact with the eukaryotic 60S ribosomal subunit and prevent polypeptide chain initiation or elongation (Carter and Cannon, 1977; Pestka, 2007; Ueno, 1984). The present study did not include a pair-fed group (i.e., a group consuming the same amount of feed as that consumed by the DON groups), and thus it was not possible to distinguish the effects of feed restriction (caused by DON) from other effects of DON that might have decreased susceptibility to *Y. ruckeri*.

The intake of DON has been reported to lead to the upregulation of cytokine levels, especially pro-inflammatory cytokines (*IL*-6, *IL*-8 and *IL*-1*β*), in several studies (piglets, (Bracarense *et al.*, 2012)); human intestinal Caco-2 cells (Maresca *et al.*, 2008; Van De Walle *et al.*, 2008); and mice (Azcona-Olivera *et al.*, 1995)). Intestinal upregulation of pro-inflammatory cytokines may explain the higher resistance of DON-treated fish to infection with *Y. ruckeri*. However, as explained by Grenier and Applegate (2013), DON, as a protein synthesis inhibitor, might naturally originate superinduction phenomena, consequently increasing cytokine synthesis and secretion. Nonetheless, the possible role of DON in the upregulation of pro-inflammatory cytokines and the consequent effect on immune stimulation should be further investigated.

2.5 Conclusions

The present findings reinforce those from previous studies, concluding that the ingestion of DON by trout over short-term periods at high dosages (50 days; 1,166 µg kg $^{-1}$ and 2,745 µg kg $^{-1}$) impacts growth performance, especially feed intake, with minor or variable biochemical changes in trout blood and histopathological changes. In this case, some fish did exhibit clinical symptoms (i.e., anal papilla), which could be attributed to the DON treatment; however, further confirmation is needed. This is the first report of the effects of the long-term exposure of rainbow trout to low concentrations of DON (168 days; 367 µg kg $^{-1}$ DON). Ingestion of DON in the long-term study was asymptomatic; however, the fish started to reduce their growth performance 92 days after ingesting DON. Such low contamination levels, which might be unnoticed by farmers, may have economic consequences for aquaculture.

DON-treated fish showed higher resistance to infection with *Y. ruckeri*, which may be related to stimulation of the pro-inflammatory response. While higher resistance to pathogen infection may be considered as a positive effect, the reduced feed intake and

lower growth performance may have economic consequences for aquaculture. Moreover, further investigation is needed to understand the influence of DON on pro-inflammatory responses.

The high levels of individual variability observed in the blood biochemical parameters, histological changes and clinical signs in the fish that were fed DON might be explained by individual intestinal microbiota composition. The individual gut microbiome and its apparent capacity to metabolize DON should be further explored.

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CHAPTER 3

Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism

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Abstract

The impact of deoxynivalenol (DON) on rainbow trout, *Oncorhynchus mykiss*, is mainly characterised by impaired growth performance and reduced feed intake, usually with the total absence of any visible clinical signs. Despite the high concentrations of DON in the present study (up to 11,412 ± 1,141 µg kg $^{-1}$), no clinical signs (except anorexia at the higher DON dosage) were observed, which confirms the difficulties of diagnosing DON ingestion. Compared to the control group, the proteolytic enzyme activities (pepsin, trypsin and chymotrypsin) in trout were altered by DON ingestion. However, it was not clear if the observed impact on digestive enzymes was due to the direct action of DON, or a consequence of the lower feed intake determined for DON-treated animals. The impact of DON on the abundance of specific measured mRNA transcripts was unexpected with higher expression levels for insulin-like growth factors, *igf1* and *igf2*, which are directly related to elevated insulin levels in plasma. This can also in part be influenced by the trypsin activity and by *npy*, given its higher mRNA expression levels. The apparent digestibility of dry matter, protein and energy was not affected by dietary levels of DON, however, nutrient retention, protein, fat and energy retention were significantly affected in animals fed DON. Adenylate cyclase-activating polypeptide (PACAP) expression seems to play an important role in controlling feed intake in DON fed trout. In the present study, we have shown for the first time that DON is metabolized to DON-3-sulfate in trout. DON-3-sulfate is much less toxic than DON, which helps to explain the lack of clinical signs in fish fed DON. Being a novel metabolite identified in trout makes it a potential biomarker of DON exposure. Suppression of appetite due to DON contamination in feeds might be a defense mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON.

Keywords: *Fusarium* mycotoxins, *Oncorhynchus mykiss*, PACAP, DON-3-sulfate, biomarker

3.1 Introduction

Mycotoxins, toxic secondary metabolites produced by moulds (Hussein and Brasel, 2001), can be produced on agricultural commodities before and/or after harvest, during transportation or storage. Mycotoxins are a significant problem worldwide causing adverse health outcomes when consumed by humans and animals (Zain, 2011) and are responsible for significant economic losses worldwide due to condemned agricultural products (CAST, 2003; Shane and Eaton, 1994; Vasanthi and Bhat, 1998). The presence of mycotoxins in aquaculture are not novel. The first report of mortality due to mycotoxicosis in aquaculture was in the early 1960s, where in the United States, hatchery-reared rainbow trout (*Oncorhynchus mykiss*) were fed cottonseed meal contaminated with aflatoxins (Kumar *et al.*, 2013; Wolf and Jackson, 1963).

In recent years, the awareness of mycotoxin-related issues in the industry has been raised again, mainly due to the increasing inclusion levels of plant meals in aquafeeds (Anater *et al.*, 2016; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017; Hooft and Bureau, 2017; Hooft *et al.*, 2011). Feed manufacturers and producers realise the importance of mycotoxins and their potential negative effects on production. However, there are still two major constraints entrenched in the aquaculture industry that make it difficult to develop an effective mycotoxin management plan for the sector.

The first constraint is the perception that the majority of mycotoxin issues stem from poor storage conditions. Poor storage conditions can lead to the growth of *Aspergillus spp*. and *Penicillium spp*., which can ultimately lead to the production of aflatoxin (AF) and ochratoxin A (OTA). In countries where climate conditions are favourable to the growth of *Aspergillus spp*. and *Penicillium spp*. fungi, optimal storage conditions should prevent the contamination of raw materials and finished feeds with AF or OTA. However, the same is not true for *Fusarium spp*., which on the contrary to *Aspergillus spp*. and *Penicillium spp*., are more likely to grow in the crops pre-harvest. The *Fusarium* mycotoxins (Type B and A trichothecenes and fumonisins) are reasonably stable to processing conditions (Cheli *et al.*, 2013). Therefore, these mycotoxins are not destroyed during raw material processing or aquafeed manufacturing, and will consequently be present in the finished feed. This may lead to potential harm to fish and shrimps, dependent upon concentration and co-occurrence. We have previously reported

that soybean meal, wheat, wheat bran, maize, corn gluten meal, rapeseed/canola meal and rice bran in samples from Asia were mostly contaminated with *Fusarium* mycotoxins (zearalenone, ZEN; deoxynivalenol, DON; and fumonisin B1, FB_1) (Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Thus, finished feed samples were mainly contaminated with *Fusarium* mycotoxins, reflecting the use of plant meals in the finished feeds and not resulting from poor storage conditions (Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Moreover, the presence of secondary metabolites of *Fusarium spp*. are expected to increase as a response to climate change (Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011), which will likely further impact the global aquaculture industry. Among the metabolites produced by the *Fusarium* genus, DON is reported as the main mycotoxin found especially in small grain cereals (Rodrigues and Naehrer, 2012; Simsek *et al.*, 2013). Despite the fact that the toxic effects and toxicokinetics of DON - a mycotoxin commonly known as "vomitoxin" as it causes vomiting in livestock - is well described including clinical symptoms for land farmed animals (Pestka, 2007), very little is known for aquatic animals.

The second constraint for mycotoxin research in aquaculture is the lack of any validated clinical symptoms in fish and shrimps when they are fed mycotoxins. In the case of DON, no known distinct subclinical signs of DON toxicoses in fish (i.e. no distinct lesions/pathologies) are described for aquaculture species. Several reports describe some clinical signs for the most common mycotoxins (see the review from Anater *et al*. (2016)), however, they are generalised and could be attributed to any diverse pathologies or challenges e.g. anti-nutrition factors or lectins in the diet (Hart *et al.*, 2010). Two notable exceptions are aflatoxicosis (yellowing of the body surface, (Deng *et al.*, 2010) and ingestion of FB (alteration of the sphinganine to sphingosine ratio, (Tuan *et al.*, 2003). Most reported clinical manifestations due to mycotoxin ingestion are related to a reduction in growth performance, alteration of blood parameters (erythrocyte/leucocyte count), blood enzymes (Alanine Aminotransferase (ALT), Aspartate Transaminase (AST) or Alkaline Phosphatase (ALP)), liver alterations or the suppression of immune parameters. Moreover, it is generally observed that the biological effects of mycotoxins vary greatly over different species. Even in the same species, they depend on the concentration of the toxin in feed, the age of the animal, and its nutritional and health status prior to mycotoxin ingestion (Hendricks, 1994).

The present work aims to evaluate and elucidate the impact of DON on rainbow trout, by exploring new tools and evaluating new diagnostic factors, which may be used later by the industry as standards to better diagnose mycotoxicoses in fish. Reduced growth performance is one of the most prevalent clinical signs of DON across fish species. Building upon the current knowledge of the impact of DON in rainbow trout (Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2015; Ryerse *et al.*, 2015), we have attempted to characterize reduced growth performance by exploring the impact of DON on ingredient digestibility. This was achieved by measuring total pepsin, trypsin, chymotrypsin, amylase and lipase activities. The expression level of gene markers for stress regulation were assessed at head kidney (*star*) and brain (*crf1, crf2, crfbp*). For growth control, liver was evaluated for *igf1* and *igf2*, and brain for *adcyap1a* (PACAP). Digestion regulation and appetite control was evaluated by examining th expression of *sst2*, *chia*, *pga*, *lpl*, *ghrl*, *cel1*, *cel2*, *cckt*, *cckn*, *cckl*, *amy2a1*, *atp4a*, *crtl*, *try1*, *try2* and *try3* on gastrointestinal tract (GIT) and *lep* and *npy* in brain. Moreover, due to DON-related damage of the GIT, faeces was analysed for DON metabolites in order to study the DON metabolism.

3.2 Material and Methods

3.2.1 Experimental diets

The trial comprised three dietary treatments (Table 3.1), all based on a single dietary formulation. The control diet (CTRL) contained a commercially relevant level of fishmeal (Fishmeal Super Prime = 12.45%; Fishmeal 60 = 14.0%) and moderate levels of plant ingredients such as soy protein concentrate, wheat gluten, corn gluten, wheat meal, soybean meal and corn meal as protein sources. Fish oil was used as the main lipid source. This control diet served also as the base of two additional diets which were supplemented, at the mixing step, with culture material extract containing DON (Romer Labs, Tulln, Austria) at 4.5 and 10.5 mg kg^{-1} (diets DON 5 and DON 11, respectively). Contamination levels were chosen taking into account previous literature (Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Matejova *et al.*, 2015; Ryerse *et al.*, 2015) instead of reported DON levels in European finished feed samples (Gonçalves *et al.*, 2018d). This because, reports of mycotoxin occurrence in European aquaculture finished feeds are still very limited, and levels reported (average DON contamination of 165.61 µg kg^{-1} (Gonçalves *et al.*, 2018d) might be underestimated.

All diets were isonitrogenous (crude protein, 52.2% dry matter (DM)), isolipidic (17.9% DM) and isoenergetic (gross energy, 22.2 MJ kg $^{-1}$ DM).

3.2.2 Manufacture of diets

Diets were manufactured by extrusion (pellet size = 2.0 mm) at SPAROS (Portugal) using a pilot-scale BC45 twin-screw extruder (CLEXTRAL, France) with a screw diameter of 55.5 mm; the operating temperature was 113–116 °C. Upon extrusion, feeds were dried in a vibrating fluid DR100 bed dryer (TGC Extrusion, France). Pellets were allowed to cool to room temperature before application of the oil fraction by coating under vacuum conditions (PG-10VCLAB instrument; DINNISEN, The Netherlands). The target amount of oil for the post-extrusion coating procedure was sprayed onto the pellets under vacuum (760 mbar) for approximately two minutes. The experimental feeds were stored in a cool and aerated location throughout the trial. Samples of each diet were taken for proximate composition analysis (Table 3.1). A sample of each diet was tested for target mycotoxin presence (DON; Table 3.1) plus other relevant mycotoxins and metabolites (Table 3.1). The natural presence of other major mycotoxins, were determined analytically as described previously and reported in Table S3.1 (Streit *et al.*, 2013).

Table 3.1: Formulation and composition of experimental diets.

Ingredients, %	**CTRL**	**DON 5**	**DON 11**
Fishmeal 60[1]	14.00	14.00	14.00
Fishmeal Super Prime[2]	12.45	12.45	12.45
Soy protein concentrate[3]	15.00	15.00	15.00
Wheat gluten[4]	12.30	12.30	12.30
Corn gluten meal[5]	8.00	8.00	8.00
Soybean meal[6]	6.00	6.00	6.00
Wheat meal[7]	6.40	6.40	6.40
Corn meal[8]	10.00	10.00	10.00
Fish oil[9]	10.00	10.00	10.00
Soy lecithin[10]	2.00	2.00	2.00
Antioxidant[11]	0.30	0.30	0.30
Monocalcium phosphate[12]	1.50	1.50	1.50
L-lysine[13]	0.50	0.50	0.50
DL-methionine[14]	0.50	0.50	0.50
Vitamin E[15]	0.05	0.05	0.05

Vitamin and mineral premix[16]	1.00	1.00	1.00
Chromium oxide, % DM	0.96	0.96	0.96
Deoxynivalenol (mg kg $^{-1}$)	--	4.5	10.5
Proximal composition			
Dry matter (DM), %	91.7 ± 0.0	91.5 ± 0.0	91.6 ± 0.0
Crude protein, % DM	52.2 ± 0.1	52.2 ± 0.0	52.2 ± 0.0
Crude fat, % DM	17.9 ± 0.0	17.8 ± 0.0	17.8 ± 0.0
Ash, % DM	9.3 ± 0.0	9.3 ± 0.0	9.3 ± 0.0
Gross energy, kJ g $^{-1}$ DM	22.2 ± 0.0	22.2 ± 0.0	22.2 ± 0.0
Chromium oxide, % DM	0.96 ± 0.02	0.96 ± 0.01	0.95 ± 0.01
Analysed mycotoxin concentrations (ppb = µg kg $^{-1}$)			
Deoxynivalenol	37.42 ± 0.0	4,714 ± 566	11,412 ± 1,141
Zearalenone	78.63 ± 0.0	78.63 ± 0.0	78.63 ± 0.0
Fumonisin B_1	67.73 ± 0.0	67.73 ± 0.0	67.73 ± 0.0
Sum of ergot alkaloids	Not detected	Not detected	Not detected
Aflatoxin B_1	Not detected	Not detected	Not detected

[1] COFACO 60: 62.3% crude protein (CP), 8.4% crude fat (CF), COFACO, Portugal; [2] Super Prime: 67.4% CP, 8.2% CF, EXALMAR, Peru; [3] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [4] VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France; [5] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [6] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; [7] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal; [8] Corn meal: 8.1% CP; 3.7% CF, Casa Lanchinha, Portugal; [9] SAVINOR, Portugal; [10] Lecico P700IPM, LECICO GmbH, Germany; [11] Paramega PX, Kemin Europe NV, Belgium; [12] MCP: 22% P, 18% Ca, Fosfitalia, Italy; [13] Lysine HCl 99%, Ajinomoto Eurolysine SAS, France; [14] DL-Methionine 99%, EVONIK DEGUSSA GmbH, Germany; [15] ROVIMIX E50, DSM Nutritional Products, Switzerland; [16] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

3.2.3 Fish and rearing conditions

All procedures involving fish were performed according to the EU guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Quadruplicate groups of 50 rainbow trout (*Oncorhynchus mykiss*), with a mean ± standard deviation (s.d.) initial body mass (IBM) of 2.52 ± 0.03 g, were fed one of the three experimental diets for 60 days. Fish were grown in quadrangular flat-bottom fish tanks (V = 250 L) supplied with well freshwater in a flow-through system; 14.3 ± 0.4 °C water temperature, 8.1 ± 0.7 mg L $^{-1}$ dissolved oxygen, and a 14 hour light : 10 hour dark photoperiod regime. Fish were hand-fed to visual satiety three times per day (twice during weekends) with utmost care to avoid feed wastage and allow a precise quantification of feed intake. Furthermore, observing feeding behaviour allowed us to

confirm that the reduced feed intake in DON fed treatments were established progressively after a certain time accepting the feeds, excluding the possibility that reduced food intake due to modified organoleptic properties of the feed. Fish were anaesthetized with 2-phenoxyethanol (200 mg L^{-1}) for group weighing at the start of the trial (day 0), at day 29, and at day 60.

3.2.4 Biological sampling

The whole-body proximate composition was analysed from a pool of ten fish sampled and stored (-20 °C) at the beginning of the trial (day 0) and from a pool of three fish per tank sampled and stored (-20 °C) at the end of the trial (day 60). Additionally, at the end of the growth trial, ten fish per tank were anaesthetized with 2-phenoxyethanol (200 mg L^{-1}) and a blood sample was collected by puncturing the caudal vein with a heparinised syringe. Blood samples were centrifuged at 1,590 ×g for ten minutes and the resulting supernatant fraction, i.e. the plasma, was transferred to a clean vial, snap-frozen in liquid nitrogen, and stored at -80 °C for the subsequent analysis of metabolites. Immediately after blood collection, fish were euthanized by anaesthetic overdose (prolonged exposure to 2-phenoxyethanol (200 mg L^{-1})) and their livers were dissected and weighed for calculating the hepatosomatic index (HSI). Moreover, samples of stomach, liver, pancreas and intestine (n=10/treatment) were preserved in liquid nitrogen and stored at –80 °C for enzyme activity measurement. Samples of the brain, GIT, liver and head kidney (n=10/ treatment) were flash frozen in liquid nitrogen, with the exception of brain samples which were preserved in RNAlater® (Invitrogen Life Technologies) for gene expression analysis. All samples were stored at –80 °C until processed. Sample collection was done randomly three hours after the animals were fed.

3.2.5 Biochemical composition of feeds, whole fish, and faeces

Analyses of feed, whole fish, and faeces were carried out with analytical duplicates following the methods described by the Association of Official Analytical Chemists (AOAC, 2006). Dry matter was measured after drying at 105 °C for 24 hours. Total ash was analysed by combustion (550 °C for six hours) in an L9/11/B170 muffle furnace (NABERTHERM, Germany). Crude protein (N × 6.25) was analysed by flash combustion followed by gas chromatographic separation and thermal conductivity detection with a Leco N FP-528 analyser (LECO Corporation, MI, USA). Crude lipid was determined by petroleum ether extraction (40–60 °C) using a SOXTEC™ 2055 Fat

Extraction System (Denmark). Gross energy was measured in an adiabatic C2000 basic bomb calorimeter (WERKE, Germany), and chromium concentration in feeds and faeces was determined by atomic absorption spectrometry in a SpectrAA 220 FS instrument (VARIAN, CA, USA) (Reis *et al.*, 2008).

3.2.6 Mycotoxin analyses in feed

The analyses were carried out as described by Streit *et al*. (2013). All samples were analysed with liquid chromatography tandem mass spectrometry (LC-MS/MS). For the purpose of data analysis, non-detect levels were based on the limit of detection (LOD) of the LC-MS/MS. The LOD for aflatoxin B_1, was 0.3 µg kg^{-1}. For ZEA, DON and OTA, the LODs were 10, 50 and 0.2 µg kg^{-1}, respectively. For FUM the LOD was 25 µg kg^{-1} for FB_1, FB_2, FB_3, and FB_4. The LOD for the remaining toxins/metabolites was 0.5 µg kg^{-1}.

3.2.7 Apparent digestibility measurements and mycotoxin analysis in fish faeces

At the end of the growth performance trial, and following the sampling procedures described in sub-chapter 3.2.3, ten fish per tank were used to determine the apparent digestibility coefficients (ADC) of dry matter, protein, and energy using the indirect method. An inert tracer (0.96% Chromium oxide, Cr_2O_3) was added to the feed and the nutrient to tracer ratio in feed and faeces were used for digestibility measurements. Faeces samples were collected using the apparatus for continuous faeces collection by filtration described by Choubert *et al*., (1979). Over the course of one week, faeces was removed from filters three hours after each feeding and stored at -20 °C. Faeces collected per tank was pooled per treatment and stored at -20 °C for subsequent analysis. Faeces was also analysed for the presence of DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate). Briefly, four 500 mg samples were extracted in duplicate three times from each treatment using 5 ml of 50% methanol. The samples were vortexed and shaken for 30, 20 and 10 minutes. The extraction samples were centrifuged – first and second centrifugations at 3200 rpm for five minutes, and the final centrifugation at 4200 rpm for five minutes - and the supernatants combined, vortexed and centrifuged again at 4200 rpm for five minutes. Prior to LC-MS/MS analysis, samples were diluted in vials (1:1) with the extraction solvent as described by Schwartz-Zimmerman *et al*. (2015).

3.2.8 Enzyme activity analyses

Enzyme extracts were prepared for enzyme activity measurement from samples previously preserved in liquid nitrogen and stored at -80 °C. Stomach and intestine samples were dissected and homogenised separately. Samples were manually homogenised in 3 mL distilled water and centrifuged for ten minutes at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from the stomach samples were measured for pepsin activity, and the supernatants from the intestine samples were analysed for trypsin, chymotrypsin, amylase and lipase activities.

Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1 mL of 0.5% acid-denatured bovine haemoglobin diluted in 0.2 M HCl-Glycine buffer (pH 3). After incubation at 37 °C for 30 minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C for 15 minutes and then centrifuged at 12000 rpm for 15 minutes. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA. For alkaline protease activities, trypsin activity was assayed using BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate. 0.5 mM BAPNA was dissolved in 1 mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing 20 mM $CaCl_2$. Chymotrypsin activity was determined using 0.2 mM SAPNA (N-succinyl-L-Ala-L-Pro-Phe-p-nitroanilide) dissolved in 1 mL DMSO and then made up to 100 mL in the same buffer. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA).

Lipase activity was measured following the method described by Versaw *et al*. (1989), with some modifications. The assay mixture contained 60 µL of 100 mM sodium taurocholate, 540 µL of 50 mM Tris-HCl, pH 8.5, 10 µL of enzyme extract and 6 µL of β-Naphthyl caprilate. The reaction was maintained for 25 minutes at 37 °C and after this time, 6 µL of 100 mM Fast Blue BB in DMSO was added before being incubated at 37 °C for five minutes. The reaction was then stopped with 60 µL TCA 0.72 N. Finally, 815 µL of 1:1 (v:v) ethyl acetate/ethanol solution was added and the absorbance recorded at 540 nm.

Amylase activity was determined by the 3, 5-dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 2% soluble starch as a substrate. 30 µL of enzyme extract and 300 µL of substrate were incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 150 µL DNS and was heated in boiling water for 10 minutes. Then, after cooling in ice, 1.5 mL of distilled water was added to the mixture and the absorbance was measured at 540 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after DNS.

3.2.9 Gene Expression Quantification

All the samples were individually processed for total RNA extraction using NucleoSpin® RNA kits (Macherey Nagel). An Ultra-Turrax® T25 with an S25N-8G dispersion tool (IKA®-Werke) was used to homogenise the brain tissue in a volume of homogenising buffer proportional to the mass of the tissue. The remaining samples (initially frozen in liquid nitrogen) were homogenised in three steps. First, using a mortar and a pestle, then with an Ultra-Turrax® T25 in liquid nitrogen and finally by taking a sample of less than 30 mg from the finely minced powder to process with an Ultra-Turrax® T10 with the kit homogenising buffer. Genomic DNA (gDNA) was removed via on-column DNase digestion at 37 °C for 30 minutes using rDNase (RNase-free) included in the kit. The RNA concentration was measured with a Qubit 2.0 fluorimeter and Qubit RNA BR assay kit (Life Technologies), whereas RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit (Agilent Technologies). Only samples with an RNA integrity number (RIN) greater than 8.0 were tested using real-time quantitative PCR (qPCR). Total RNA (500 ng) from each sample was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences) in a Mastercycler® proS (Eppendorf) and as previously described by Mata-Sotres *et al*. (2016). A pool of cDNA from all the samples for each tissue was used for calibration plots, using six serial 1/10th dilutions from 10 ng to 100 fg, in order to assess the linearity and efficiency of the different primer combinations, as well as for being used for inter-assay calibration. Control reactions with RNase free water (NTC) and RNA (NRT) were included in the analysis to ensure the absence of primer-dimers and genomic DNA contaminations. The linearity and amplification efficiency for each pair of primers are shown in Table S3.2. Previously, primers pairs were tested for final working concentrations (optimum 200 nM) and temperature (60 °C). qPCR reactions were performed in triplicate with 1 ng of cDNA (estimated from

the input of total RNA) forward and reverse primers (Table S3.2) for the named samples (200 nM each) and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences). Reactions were performed in a volume of 10 µL using Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). The thermocycling procedures were carried out with an initial denaturation and polymerase activation at 95 °C for ten minutes, followed by 40 cycles of denaturation for 15 seconds at 95 °C, annealing and extension at 60 °C for 30 seconds, and finishing with a melting curve from 60 °C to 95 °C, increasing by 0.5 °C every five seconds. Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer–dimer artifacts. Relative gene expressions were quantified in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the ΔΔCT method (Livak and Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalised by geometric averaging of two references genes (Vandesompele *et al.*, 2002), *actb* and *ef1a*. The genes were selected owing to their lower than 0.5 target stability M value and lower than 0.25 CVs (as indicated by BioRad CFX Manager Target Stability Value). A pool of all the cDNA samples was used as a calibrator on every qPCR plate to correct for inter-assay differences. qPCR reactions were carried out with 10 ng of cDNA (assumed from RNA input), forward and reverse primers, and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences) in a final volume of 10 µL. qPCR primer sequences, amplicon sizes, amplification efficiencies, R^2 and GenBank accession number of the sequences are shown in Table S3.2. RNA nucleotide sequences for *ef1a*, *actb*, *star*, *igf1*, *igf2*, *crf1*, *crf2*, *crfbp*, *npy*, *adcyap1a*, *lep*, *sst2*, *chia*, *pga*, *lpl* and *ghrl*, were obtained from GenBank, and nucleotide sequences for *cel*, *cel2*, *amy2a*, *atp4a*, *crtl*, *try1*, *try2* and *try3* were retrieved from the *Oncorhynchus mykiss* WGS project database, using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer/).

3.2.10 Growth, feed intake, digestibility, and nutrient budget calculations

IBW (g): Initial mean body weight

FBW (g): Final mean body weight

Specific growth rate (SGR; % day $^{-1}$) = (Ln FBW – Ln IBW) × 100 days $^{-1}$

Feed conversion ratio (FCR) = crude feed intake / weight gain

Feed intake (FI; % BW day $^{-1}$) = (crude FI (DM feed) / (IBW + FBW) / 2 / days) × 100

Protein efficiency ratio (PER) = wet weight gain / crude protein intake

Hepatosomatic index (HSI) = (liver weight / body weight) × 100

Nutrient retention (%) = ((FBW × NFF) – (IBW × NIF))/Nutrient intake × 100,

with NFF being the nutrient content of final fish and NIF the nutrient content of initial fish.

Apparent digestibility coefficient (ADC, %) = 100 × [1 – (% Cr_2O_3 feed / Cr_2O_3 faeces) × (% nutrient faeces / % nutrient feed)]

Daily average nitrogen (N) gain = ((final body N content – initial body N content) / (IBW+FBW))/ 2 / days

Daily N intake = (N intake / (IBW + FBW) / 2) / days

Daily faecal N losses = daily N intake × (100 – ADC Protein) / 100

Daily metabolic N losses = daily N intake – (daily N gain + daily faecal N losses)

3.2.11 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare differences between the three diets. A post-hoc Tukey honest significant difference (HSD) test was used when ANOVA results revealed significant differences ($p < 0.05$). The Shapiro-Wilk test was used to analyse the normality and homogeneity of variances was tested using Levene's test. Data analysed did not violate the assumption of equal variances and showed normal distribution. All parameters expressed as percentages were subjected to arcsin square root transformation. Data are presented as the mean of quadruplicates ± standard deviation. All statistical tests were performed in IBM SPSS Statistics 18 software (IBM Corp., USA).

3.3 Results

3.3.1 Zootechnical performance

3.3.1.1 After 29 days of experimental feeding

The survival rate of the rainbow trout was high (> 97.0%), across the three dietary treatments with only minor mortality in the DON 5 and DON 11 treatments, that was not significant ($p > 0.05$; Table 3.2). After 29 days, all other zootechnical parameters (FBW, g; SGR, % d^{-1}; FCR; FI, %; ABW d^{-1}; PER), were significantly affected by dietary inclusion of DON ($p < 0.001$; Table 3.2). FBW ranged from 3.90 to 9.39 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR, FI and PER than those fed both DON supplemented diets (DON 5 and DON 11) ($p < 0.001$). Additionally, fish fed

the highest DON dose (DON 11) showed a significantly lower FBW, SGR, FI and PER than those fed with the DON 5 diet ($p < 0.001$). The FCR varied between 0.96 and 1.56. Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet ($p < 0.001$).

Table 3.2: Growth performance after 29 days of feeding (IBW: 2.52 ± 0.11 g).

Diet	CTRL	DON 5	DON 11	*p* value
Survival, %	100.0 ± 0.0	99.0 ± 1.2	97.0 ± 3.5	***0.103***
FBW, g	9.39 ± 0.24 ^{c}	5.83 ± 0.25 ^{b}	3.90 ± 0.23 ^{a}	***<0.001***
SGR, % d $^{-1}$	4.53 ± 0.09 ^{c}	2.90 ± 0.16 ^{b}	1.50 ± 0.23 ^{a}	***<0.001***
FCR	0.96 ± 0.02 ^{a}	1.08 ± 0.03 ^{a}	1.56 ± 0.19 ^{b}	***<0.001***
FI, %ABW d $^{-1}$	3.81 ± 0.12 ^{c}	2.93 ± 0.14 ^{b}	2.13 ± 0.15 ^{a}	***<0.001***
PER	2.18 ± 0.04 ^{c}	1.93 ± 0.06 ^{b}	1.35 ± 0.17 ^{a}	***<0.001***

Values are means and standard deviation (n = 4).
Different superscripts within a row denotes a statistical difference ($p < 0.05$).

3.1.2.1 After 60 days of experimental feeding

After the second experimental period, mortality was observed in all treatments (Table 3.3). The survival rate of the fish fed CTRL and DON 5 diets was significantly higher than that observed in fish fed the DON 11 diet ($p = 0.002$). Fish from the best performing treatment (CTRL) had a 9.8-fold increase of their initial body mass, with FBW across each of the treatments ranging from 5.96 to 24.77 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR and FI than those fed both DON supplemented diets (DON 5 and DON 11; $p < 0.001$). Additionally, fish fed the highest DON dose (DON 11) showed a significantly lower FBW, SGR and FI than those fed with the DON 5 diet ($p < 0.001$). Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet ($p = 0.001$). The HSI varied between 1.62 and 2.47, with CTRL fish showing a significantly lower HSI than those fed both DON supplemented diets (DON 5 and DON 11; $p < 0.001$; Table 3.3). Rainbow trout's differences in growth between the three dietary treatments were well visible (Figure 3.1; Control, DON 5 and DON 11, from left to right). Fish shown in Figure 3.1 are examples of the growth difference found in the different experimental groups. Despite the differences in growth, no clinical signs are observed, except the accentuated anorexia in DON 11 (far right, Figure 3.1).

Table 3.3: Growth performance after 60 days of feeding (IBW: 2.52 ± 0.11 g).

Diet	CTRL	DON 5	DON 11	*p* value
Survival, %	99.5 ± 1.0 [b]	98.0 ± 2.3 [b]	94.0 ± 2.8 [a]	***0.020***
FBW, g	24.77 ± 0.86 [c]	12.52 ± 0.83 [b]	5.96 ± 0.54 [a]	***<0.001***
SGR, % d^{-1}	3.81 ± 0.06 [c]	2.67 ± 0.12 [b]	1.43 ± 0.15 [a]	***<0.001***
FCR	1.07 ± 0.03 [a]	1.12 ± 0.03 [a]	1.58 ± 0.23 [b]	***0.001***
FI, %ABW d^{-1}	2.90 ± 0.10 [c]	2.47 ± 0.11 [b]	1.99 ± 0.16 [a]	***<0.001***
PER	1.96 ± 0.06 [b]	1.88 ± 0.06 [b]	1.35 ± 0.18 [a]	***<0.001***
HSI, %	1.62 ± 0.07 [a]	2.47 ± 0.07 [b]	2.19 ± 0.30 [b]	***<0.001***

Values are means and standard deviation (n = 4).
Different superscripts within a row denotes a statistical difference ($p < 0.05$).

Figure 3.1: Rainbow trout's visual differences in growth between the three dietary treatments (Control, DON 5 and DON 11, from left to right). Fish shown in figure are examples of the growth difference found in the different experimental groups. No clinical signs are observed, except the accentuated anorexia in DON 11 (far right).

3.3.2 Whole-body composition

The whole-body composition of fish in terms of moisture, fat and energy was not affected by the various dietary treatments ($p > 0.05$, Table 3.4). However, fish fed the CTRL diet showed a significantly lower whole-body ash and a significantly higher whole-body protein than those fed both DON supplemented diets (DON 5 and DON 11; $p < 0.001$).

Table 3.4: Whole-body composition.

Diet	CTRL	DON 5	DON 11	*p* value
Moisture, %	63.53 ± 0.95	63.73 ± 0.78	64.36 ± 0.64	***0.353***
Protein, %	16.67 ± 0.52 [b]	15.22 ± 0.45 [a]	15.09 ± 0.24 [a]	***0.001***
Fat, %	14.06 ± 0.65	13.95 ± 0.28	13.65 ± 0.52	***0.534***
Ash, %	5.05 ± 0.26 [a]	6.03 ± 0.18 [b]	6.13 ± 0.09 [b]	***<0.001***
Energy, kJ g $^{-1}$	8.50 ± 0.26	8.51 ± 0.15	8.46 ± 0.29	***0.953***

Initial fish values: moisture 65.30%; protein 17.14%; fat, 11.97%; ash 5.11%; energy 6.63 kJ g $^{-1}$.
Values are means and standard deviation (n = 4), after 60 days of feeding.
Different superscripts within a row, denotes a statistical difference ($p < 0.05$).

3.3.3 Whole-body nutrient retention and apparent digestibility

Protein retention varied between 17.83 and 32.56%, and fish fed the CTRL diet showed a significantly higher protein retention than those fed both DON supplemented diets (DON 5 and DON 11) ($p < 0.001$; Table 3.5). Moreover, fish fed the highest DON dose (DON 11) showed a significantly lower protein retention than those fed with the DON 5 diet ($p = 0.001$). Fat retention ranged from 58.98 to 81.86%, while energy retention varied between 31.09 and 40.16%. Fish fed the CTRL and DON 5 diets showed a significantly higher fat and energy retention than those fed the DON 11 diet ($p < 0.001$; Table 3.5). Digestibility of dry matter, protein and energy was not affected by dietary treatments ($p > 0.05$; Table 3.6).

Regarding, DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) analysed in trout's faeces, only DON and DON-3-sulfate were detected (Figure 3.1). No DON or DON metabolites were detected in animals fed the control diet (CTRL). In the faeces from treatment DON 5 and DON 11, 13.2% and 10.5% of DON was recovered, respectively, compared to the total ingested DON. The high solubility of DON in water may explain such low recovery rates. More than 80% of the recovered mycotoxin in faeces, was in the form of DON-3-sulfate (DON 5 = 84.07% and DON 11 = 82.09% of DON-3-sulfate) and around of 15% of DON (DON 5 = 15.93% and DON 11 = 17.91% of DON).

Table 3.5: Whole-body nutrient retention.

Diet	CTRL	DON 5	DON 11	*p* value
Protein, %	32.56 ± 1.21 [c]	27.40 ± 1.03 [b]	17.83 ± 2.40 [a]	***<0.001***
Fat, %	81.86 ± 4.32 [b]	78.98 ± 0.64 [b]	58.98 ± 10.59 [a]	***0.001***
Energy, %	40.16 ± 0.68 [b]	39.41 ± 1.15 [b]	31.09 ± 3.59 [a]	***0.001***

Values are means and standard deviation (n = 3), after 60 days of feeding.
Different superscripts within a row denotes a statistical difference ($p < 0.05$).

Table 3.6: Apparent digestibility of dry matter, protein and energy.

Diet	CTRL	DON 5	DON 11	*p* value
Dry matter, %	70.0 ± 3.1	68.7 ± 1.7	68.4 ± 1.8	***0.576***
Protein, %	89.1 ± 1.2	88.5 ± 0.7	88.4 ± 0.7	***0.499***
Energy, %	82.2 ± 2.0	81.5 ± 1.1	81.3 ± 0.4	***0.621***

Values are means and standard deviation (n = 4), after 60 days of feeding.

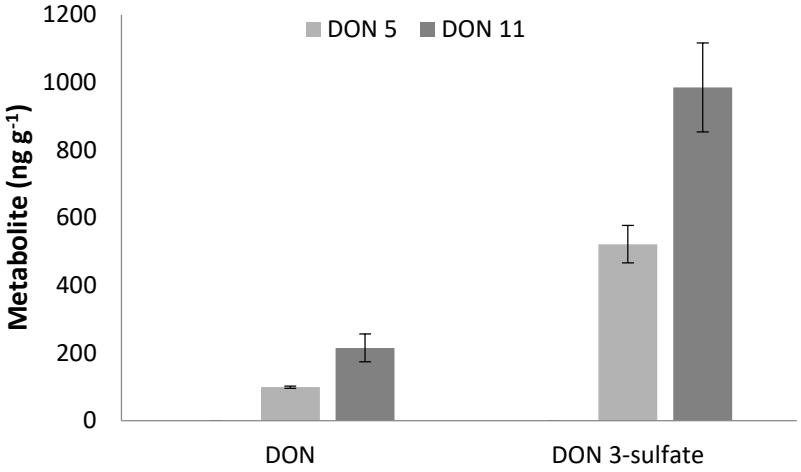

Figure 3.2: Quantity of DON and DON 3-sulfate in ng per g of faeces for treatment DON 5 and DON 11. No DON or DON-metabolites were found in the control treatment, after 60 days of feeding. DOM and DOM 3-sulfate were not identified in the samples collected. Values are means and standard deviation of the mean (n = 8).

3.3.4 Enzyme Activity Analyses

The results of total pepsin, trypsin, chymotrypsin, amylase and lipase activities measured in the different experimental groups are shown in Figure 3.2 (i to v, respectively). For total pepsin, DON 11 showed a higher activity (0.0021 ± 0.0003 U g $^{-1}$ BW; $p < 0.001$) when compared to the CTRL (0.0012 ± 0.0002 U g $^{-1}$ BW) and DON 5 (0.0013 ± 0.0003 U g $^{-1}$ BW) groups. In contrast, trypsin, showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g $^{-1}$ BW; $p = 0.043$) being statistically lower than DON 11 (2.40 ± 1.19 U g $^{-1}$ BW), however, statistically similar ($p > 0.05$) to the control (2.58 ± 2.08 U g $^{-1}$ BW). Chymotrypsin did not present significant differences ($p > 0.05$) among the different experimental groups. DON 11 presented a higher lipase activity (0.0022 ± 0.0001 U g $^{-1}$ BW; $p = 0.002$) compared to CTRL (0.0005 ± 0.00001 U g $^{-1}$ BW) and DON 5 (0.0013 ± 0.0007 U g $^{-1}$ BW). Amylase did not present significant differences ($p > 0.05$) among the different dietary groups.

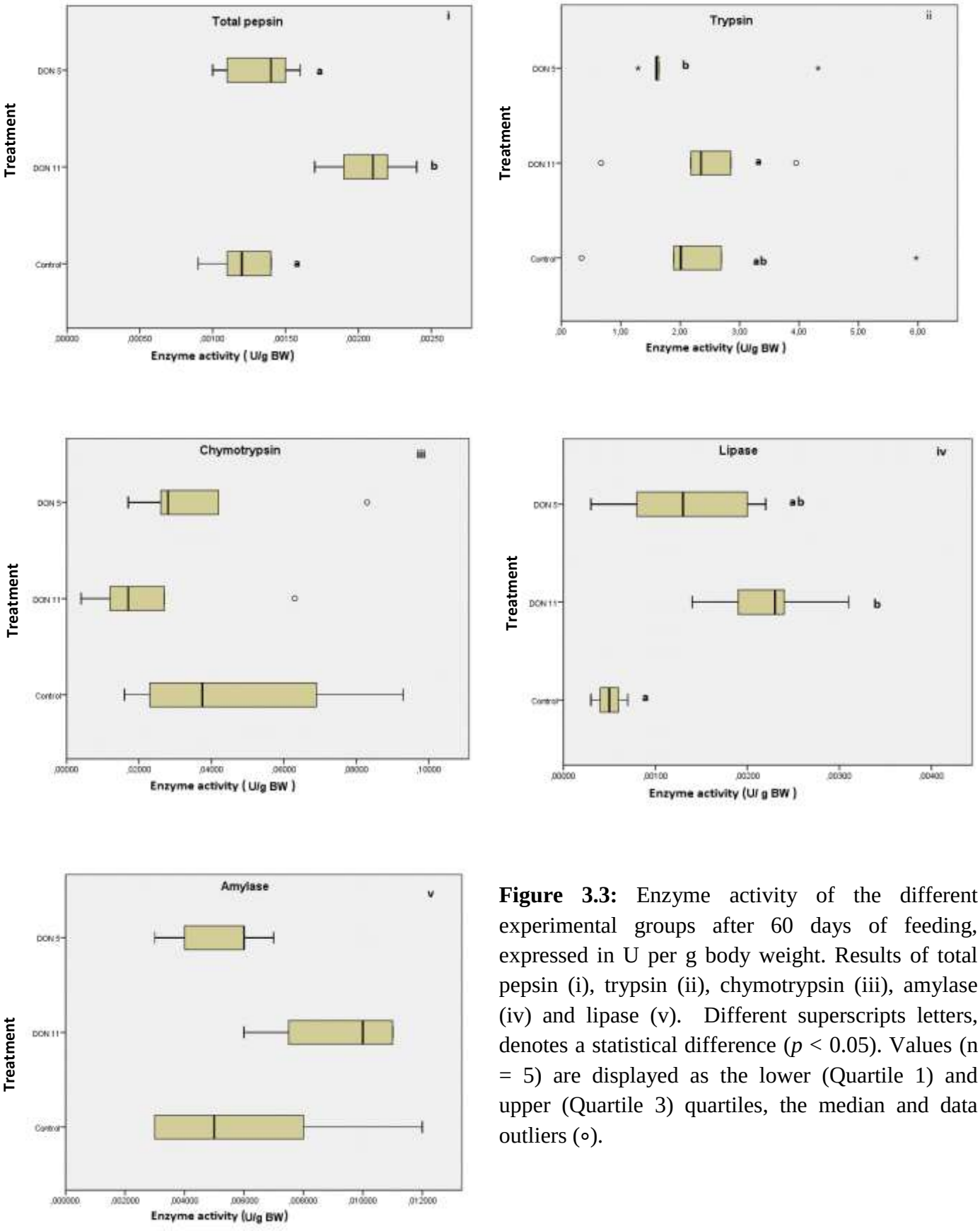

Figure 3.3: Enzyme activity of the different experimental groups after 60 days of feeding, expressed in U per g body weight. Results of total pepsin (i), trypsin (ii), chymotrypsin (iii), amylase (iv) and lipase (v). Different superscripts letters, denotes a statistical difference ($p < 0.05$). Values (n = 5) are displayed as the lower (Quartile 1) and upper (Quartile 3) quartiles, the median and data outliers (∘).

3.3.5 Gene Expression

The relative expression levels of gene transcripts for stress regulation (*star*; head kidney; *crf1*, *crf2*, *crfbp*; brain), growth control (*igf1*, *igf2*; liver; adcyap1a; brain), enzymatic digestion, regulation and appetite control (*sst2*, *chia*, *pga*, *lpl*, *ghrl*, *cel1*, *cel2*, *cckt*, *cckn*, *cckl*, *amy2a1*, *atp4a*, *crtl*, *try1*, *try2*, *try3*; GIT; *lep*, *npy*; brain) are shown in Table 3.7. Expression of *igf1* and *igf2* was significantly lower in DON 5 and DON 11 when compared to CTRL group (p = 0.004 and p = 0.008, respectively). Interestingly, expression levels of npy and *adcyap1a*/PACAP mRNAs were significantly up-regulated by DON treatments (p = 0.004 and p = 0.005, respectively). In contrast, *star* mRNA transcripts displayed a trend toward higher abundance in both DON fed treatments, however, this was not significant (p = 0.088). Analyses of specific mRNA transcript levels across all enzyme precursors (*crtl*, *Try1*, *Try2*, *Try3*), highlighted *try3* to be significantly up-regulated (p = 0.036) in both DON fed groups.

Table 3.7: Relative expression levels of genes tested for the three experimental treatments.

Tissue	Genes	CTRL	DON 5	DON 11	*p* value
Head Kidney	*star*	0.36±0.14	2.87±1.10	2.26±0.77	0.088
Liver	*Igf1*	0.95±0.16 [a]	0.43±0.08 [b]	0.49±0.04 [b]	0.004
	Igf2	0.90±0.23 [a]	0.41±0.06 [b]	0.25±0.06 [b]	0.008
Brain	*crf1*	1.03±0.07	1.19±0.26	1.22±0.16	0.726
	crf2	1.02±0.07	1.12±0.10	1.05±0.08	0.660
	crfbp	0.94±0.09	0.79±0.15	0.93±0.07	0.555
	npy	0.94±0.14 [a]	1.59±0.18 [b]	1.67±0.30 [b]	0.004
	adcyap1a	0.99±0.08 [a]	1.40±0.11 [b]	1.44±0.11 [b]	0.005
GIT	*lep*	0.94±0.09	0.95±0.17	1.11±0.18	0.640
	sst2	0.90±0.08	0.70±0.18	0.82±0.10	0.489
	chia	0.96±0.11	1.10±0.20	1.28±0.25	0.497
	pga	0.97±0.17	0.88±0.17	0.78±0.10	0.640
	lpl	1.17±0.22	1.74±0.38	1.03±0.11	0.122
	ghrl	0.88±0.19	1.54±0.34	1.20±0.16	0.127
	cel1	1.25±0.26	1.29±0.29	0.83±0.14	0.293
	cel2	0.94±0.16	0.80±0.24	0.59±0.09	0.313
	cckt	0.84±0.16	0.89±0.16	0.70±0.14	0.612
	cckn	1.25±0.28	1.35±0.15	1.83±0.39	0.291
	cckl	0.99±0.19	1.14±0.08	1.18±0.21	0.706
	amy2a	1.05±0.15	1.10±0.27	1.00±0.16	0.929

atp4a	1.00±0.14	1.41±0.27	1.09±0.16	0.278
crtl	1.06±0.21	1.01±0.19	0.68±0.10	0.228
try1	0.88±0.23	0.70±0.23	0.64±0.12	0.653
try2	1.22±0.29	1.25±0.24	0.95±0.15	0.612
try3	1.00±0.15 [a]	2.03±0.43 [b]	1.90±0.25 [b]	0.036

Values are means and standard deviation of the mean (n = 7) after 60 days of feeding.
Different superscripts within a row denotes a statistical difference ($p < 0.05$).

3.4 Discussion

The impact of DON on fish has been further elucidated in recent years with the rainbow trout as a useful model. However, very little is known in comparison to land-farmed animals. This is especially true regarding diagnostic parameters to correctly identify the impact of DON ingestion in a production setting. For this purpose, an experimental protocol was designed to further understand the impact of DON on growth performance and also to explore the underpinning causes for the reported decreases in growth performance. The DON effect was evaluated by studying the regulation of digestion, both at the enzyme activity level and through mRNA gene expression, where we surveyed stress regulation, growth and appetite control processes. In order to investigate metabolic breakdown of DON, faeces was analysed for DON metabolites.

Growth performance was affected by DON (4,714 ± 566 and 11,412 ± 1,141 µg kg $^{-1}$), in a similar manner to that previously described (Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Ryerse *et al.*, 2015), despite higher DON concentrations. Fish fed DON showed a significantly lower FBW, SGR and FI when compared to the control ($p < 0.001$). Moreover, the highest DON dose (DON 11) showed a significantly lower FBW, SGR and FI than the dose used in the DON 5 diet ($p < 0.001$). In addition, FCR was significantly increased in fish fed the DON 11 diet ($p = 0.001$). The HSI was significantly higher in animals fed DON, although this difference was not observed during dissection or visual examination for clinical signs. Interestingly, no macroscopic lesions were found (e.g. internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) were detected on animals fed DON, confirming that diagnosis of DON ingestion is extremely difficult, even at high dosages (DON 11; 11,412 ± 1,141 µg kg $^{-1}$). According to previous studies, the impact of DON might vary greatly depending on unknown factors, even for the same species. For the same range of DON contamination (0.3 to 5.9 mg kg $^{-1}$), some authors (Hooft *et al.*,

2011; Ryerse *et al.*, 2015) did not find any major pathological changes in the distal intestine of trout, while in other situations, gastrointestinal haemorrhages were found (Matejova *et al.*, 2014). In addition to the lower growth in the DON fed treatments, which was visually detected (Figure 3.1), DON 11 treated fish also had significantly lower survival rates. In our opinion, it is highly unlikely under production conditions that such an increase in mortality/decrease in performance would be associated to DON ingestion particularly when specific subclinical signs are lacking. The present scenario illustrating the significant impact of DON in trout coupled to a complete lack of clinical symptoms highlights the need for further investigation to support an early diagnosis for DON ingestion.

Anti-nutritional factors that decrease enzymatic activity, or form complexes with proteins thereby modifying digestion processes have been described (Moyano *et al.*, 1999; Santigosa *et al.*, 2008). However, very little is known about the impact of mycotoxins on digestive enzymes and information regarding the impact of DON is very scarce. For AF ingestion, Han *et al*. (2008) observed increased protease, amylase, chymotrypsin, and trypsin activity and an apparent decrease in digestibility of crude protein in 42-day old ducks fed 0.02 and 0.04 mg kg $^{-1}$ AF. To our knowledge most studies have been focused on the effects of DON on the nutrient absorption process (Grenier and Applegate, 2013), however, there is no information available regarding the effects of DON on digestive enzymes.

The major contributing factor to the conversion of feed to growth is protein turnover thus proteases play an essential role. Proteolytic enzyme activity (pepsin, trypsin and chymotrypsin) was significantly altered in DON fed groups. Total pepsin activity was significantly higher in the DON 11 group (0.0021 ± 0.0003 U g $^{-1}$ BW; $p < 0.001$) compared to the CTRL (0.0012 ± 0.0002 U g $^{-1}$ BW) and DON 5 (0.0013 ± 0.0003 U g $^{-1}$ BW) groups. However, the observed impact upon pepsin might be directly related to the decrease of feed intake in this group, and not necessarily a direct impact of DON on pepsin or any pepsin precursor. It is well described that pepsinogen is rapidly synthesised during feeding and then secreted, whereupon pepsin activity increases. For example, Einarsson *et al*. (1996) observed that under starvation conditions in *Salmo salar* there was a slight rise in pepsin activity in the stomach mucosa suggesting that pepsin can be stored in salmonids. Therefore, and taking into account that animals were fed three hours prior to sampling, we cannot exclude the hypothesis that higher pepsin

secretion in the DON 11 group could be related to lower feed intake. This in turn may result in a retention of pepsin in stomach mucus, due to a markedly reduced stomach evacuation. Chymotrypsin did not show significant differences between treatments, although a numerically lower activity was observed in the DON 11 group. Rungruangsak-Torrisen *et al*. (2006) observed a higher specific activity of chymotrypsin when growth was limited or depressed due to starvation or food deprivation. However, considering that feed intake was reduced in the DON 11 group, possibly due to hormonal regulation, no major effect was observed in chymotrypsin activity. As trypsin activates chymotrypsin in fish (Sunde *et al.*, 2001) it is difficult to find out if DON impacted, directly or indirectly, this activation. Trypsin showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g $^{-1}$ BW; p = 0.043), statistically insignificant when compared to DON 11 (2.40 ± 1.19 U g $^{-1}$ BW) but similar to CTRL (2.58 ± 2.08 U g $^{-1}$ BW). Also interesting is the observation that from the four types of alkaline proteases (*ctrl*; *try1, try2* and *try3*), only mesotrypsinogen (trypsinogen-3) showed a higher mRNA expression level in DON fed treatments. Mesotrypsin is a specialised protease known for its resistance to trypsin inhibitors. It is thought to play a special role in the degradation of trypsin inhibitors, possibly to help with the digestion of inhibitor-rich plant meals such as soybeans and lima beans, which might be the case of plant meals containing DON as well (Szmola *et al.*, 2003), however more research is needed on this topic.

Both trypsin activity and mRNA expression levels for *igf1* and *igf2* were found to be significantly higher in the CTRL when compared to DON 5 and DON 11. It is well known that trypsin cleaves protein at the carboxyl side of the basic amino acids lysine and arginine (Stryer, 1988), which elevates plasma insulin levels in salmonids (Plisetskaya *et al.*, 1991). In turn, insulin stimulates amino acid uptake and protein synthesis especially in the muscle tissue (Matty, 1986; Murat *et al.*, 1981), leading to a growth promoting effect in salmonids (Donaldson *et al.*, 1979). Proteolytic enzyme differences observed between the treatments were probably not associated with secretion control, as cholecystokinin-like peptides (*cck-t*; *cck-n* or *cck-l*) did not significantly alter due to DON treatment.

Apparent digestibility of dry matter, protein and energy in the present study was not affected by dietary levels of DON, which agrees with a previous study in trout (Hooft *et al.*, 2011). In the current literature, contradictory information about the effects of DON

on ADC has been reported. While, for example, Dänicke *et al*. (2004) and Van Le Thanh *et al*. (2015) reported that DON could affect crude protein digestibility in piglets, Jo *et al*. (Jo *et al.*, 2016) reported no differences in ADC in growing pigs fed 10,000 µg kg $^{-1}$ DON. The latter is in agreement with findings from the present study for similar levels of contamination. Jo *et al*. (2016) reported that DON contamination might affect essential amino acid digestibility. In the present study, it was observed that DON affected trypsin, and consequently, trypsin may influence the levels of insulin, which will ultimately influence amino acid uptake. However, this requires further research to verify whether DON directly affects this pathway.

In this study, protein, fat and energy retention were all significantly affected in animals fed DON. The low performance of the animals fed DON could be a consequence of decreased nutrient uptake and transport rather than lower nutrient digestibility, as enzyme activity and ADC appear unaffected by DON. The reason behind the enzyme activity differences among the experimental groups is not clear, however differential feed intake may influence our interpretation.

The neuroendocrine process that controls satiety is regulated, with others, by Neuropeptide Y, Leptin, Ghrelin or Adenylate cyclase-activating polypeptide (PACAP). In the present work, leptin and ghrelin mRNA transcripts were not influenced by DON which was not surprising as Leptin activity is related to long-term regulation of energy balance, suppressing food intake, while Ghrelin is a fast-acting hormone acting as "stopper" after meal initiation (Klok *et al.*, 2007). In contrast, PACAP plays an important and direct role in the regulation of feed intake. In goldfish, it has been observed that intracerebroventricular injections of PACAP suppress food intake (Matsuda *et al.*, 2005). In the present study, upregulation of *adcyap1a* or PACAP mRNAs provides a possible link to the observed reduction in feed intake, as described in the literature (Chance *et al.*, 1995; Li *et al.*, 2015; Morley *et al.*, 1992; Mounien *et al.*, 2008; Tachibana *et al.*, 2003). In zebrafish, PACAP greatly decreases the frequency of gut motility waves (Holmberg *et al.*, 2004) which might also have an impact on nutrient absorption. *npy* was also upregulated in DON fed treatments, however, its putative role in our experimental setup is challenging to explain. In mammals, *npy* is a key factor in the regulation of feeding behaviour and there is strong evidence of a direct physiological role of *npy* and its expression levels in controlling feed intake (Chamorro *et al.*, 2002). However, most of the studies published suggest that elevated brain *npy*

levels induce increased feed intake (see review Chamorro *et al*., 2002), which is contrary to the obtained results in this study. Though, in the present study the upregulation of *npy*, seems to be a consequence of the reduced feed intake, i.e., *npy*, as explained by Narnaware and Peter (2001), is regulated in part by the feeding state of the animal, since food deprivation induces a marked increase in both npy and its mRNA levels in the brain. Narnaware and Peter, 2001, observed an increased npy mRNA expression in several brain regions of goldfish in response to food deprivation, which might help to explain the findings of the present study.

Metabolism of DON could also explain the lack of any lesions in trout when compared, for example, with swine and poultry. Metabolism of trichothecenes in several livestock species has been reported however, these studies focus on the formation of de-epoxy-DON or on glucuronidation (Dänicke *et al.*, 2004; Eriksen *et al.*, 2002; Schwartz-Zimmermann *et al.*, 2015). While de-epoxy-DON is achieved mainly by gut microbiota, glucuronidation is carried out by endogenous UDP-glucuronosyltransferases in the liver, and possibly also in intestinal microsomes (Maul *et al.*, 2015). Metabolism pathways of DON vary greatly within species (Schwartz-Zimmermann *et al.*, 2015). In fish, only one report in brown bullhead catfish (*Ameriurus nebulosus*) has shown the capability of the gut microbiota of this species to biotransform trichothecenes into their de-epoxy forms (Guan *et al.*, 2009). DON can also be metabolized by sulfation, which was only recently discovered as a major pathway for chickens and turkeys (Devreese *et al.*, 2015; Schwartz-Zimmermann *et al.*, 2015; Wan *et al.*, 2014). In the present study, it has been shown for the first time in rainbow trout that DON is metabolized into DON-3-sulfate, which is less toxic than DON (Schwartz-Zimmermann *et al.*, 2015). Despite considerable mycotoxin leaching from the faeces, due to the high solubility of DON/DON-metabolites in water, more than 80% of the mycotoxin in faeces was recovered in the form of DON-3-sulfate. The location of formation, absorption and elimination of DON-3-sulfate is not known and was not further investigated in the present trial. However, as suggested by (Schwartz-Zimmermann *et al.*, 2015), DON might be metabolized to DON-3-sulfate in the intestinal mucosa, liver, or even in the kidney as happens for some other vertebrate species. The formation of DON-3-sulfate, could also explain the absence of major clinical signs in trout fed DON, particularly at DON 5 treatment levels, which still had a considerable impact on feed intake but did not cause major lesions (external macroscopic observations).

In the present work, the impact of DON on the GIT or on the absorptive process was not evaluated. The potential impact of mycotoxins on the GIT in livestock species is well described (see reviews; (Broom, 2015; Grenier and Applegate, 2013). Due to the mode of action of DON (as an inhibitor of protein synthesis) and the high rate of protein turnover in intestinal cells, it is to some extent also expected to observe altered intestinal areas in trout. However, the literature is not consistent when reporting the impact of DON on the trout GIT (Hooft *et al.*, 2011; Matejova *et al.*, 2014; Ryerse *et al.*, 2015). Despite only being evaluated macroscopically, any potential microscopic intestinal damage caused by DON and the consequent influence on nutrient absorption cannot be discarded. However, the novel fact that 80% of the recovered DON is metabolised into DON-3-sulfate might help to explain the lack of consistency in GIT damage in trout. The elucidation of metabolism pathways in fish in respect to DON would be a major step toward understanding the underpinning mechanisms of sensitivity/resistance to this mycotoxin in fish.

3.5 Conclusion

Deoxynivalenol exposure in fish has been characterised mainly by reduced feed intake and growth performance. Contrary to land farm animals, DON ingestion in fish does not lead to specific clinical signs, except anorexia (at high dosages, above 5 mg kg^{-1}), and some minor altered blood parameters (blood parameters (erythrocyte/leucocyte count), blood enzymes (ALT, AST or ALP), liver alterations or immune parameters suppression) which are generally not specific for DON-induced mycotoxicosis. In the present study, it was observed that digestive enzymes (regarding activity and mRNA expression) are affected; however, we were unable to clarify if this was caused by DON ingestion or by suppression of feed intake. A pair-fed treatment would be useful in the future in order to distinguish between effects from DON intake and any possible effects from suppression of feed intake. Moreover, nutrient (protein, fat and energy) retention was affected by dietary DON suggesting that nutrient uptake and transport might be affected. In the present study the upregulation of *npy*, seems to be a response to food deprivation, but in contrast upregulation of PACAP seems to be fundamental for explaining the reduction of feed intake in DON fed treatments, inducing anorexia. Further research is needed focusing on the effect of DON on appetite control by addressing the influence of DON on the hypothalamic melanocortin system. Suppression of appetite due to DON contamination in feeds might be a defence

mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The discovery of DON-3-sulfate as a novel trout metabolite makes it a potential biomarker of DON exposure. However, further characterisation of its toxicological relevance is essential.

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Contribution

The experimental design was from the responsibility of RAG, DS, GMR and SM. In vivo experiment and digestibility measurement was at the responsibility of JD and PR. Enzyme activities were performed by CN, MY and FJM. Gene expression were performed by RAG, NG, GMR. Mycotoxin metabolites were analysed by GB and TC. Results analyse, writing, statistical treatment and interpretation of the data, was the responsibility of RAG with the active collaboration of all co-authors. All authors have approved the final article and contributed for its revision.

Declaration of interest

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CHAPTER 4

Fate of [^{3}H]-deoxynivalenol in rainbow trout (*Oncorhynchus mykiss*) juveniles: tissue distribution and excretion

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Abstract

Deoxynivalenol (DON), a *Fusarium* mycotoxin, is one of the most prevalent mycotoxin in aquafeeds. The toxicokinetics of DON is rarely studied in aquatic species. The present study used juvenile rainbow trout (*Oncorhynchus mykiss*) with a mean initial body weight of 7.72 ± 1.42 g in order to evaluate the pharmacokinetic behaviour and the metabolism of radiolabelled DON ([^{3}H]-DON). In a first trial, 30 fish were tube-fed with four pellets containing a total of 125 ± 0.019 ng of [^{3}H]-DON. At different sampling time points after feeding (1 h, 3 h, 6 h, 12 h or 24 h), the tissue distribution of the [^{3}H]-DON was assessed by liquid scintillation counting. In a second trial, five fish were tube-fed four pellets containing a total of 663 ng of unlabelled-DON. Twenty-four hours after feeding, metabolites of DON excreted into the water were analysed by LC-MS/MS. [^{3}H]-DON was detected in fish liver one hour after tube-feeding, indicating a rapid absorption of DON. In the first hour, [^{3}H]-DON achieved its maximum in the gastro-intestinal tract (GIT) (20.56 ± 8.30 ng). However, 6.19 ± 0.83 ng of [^{3}H]-DON was also detected in the water at this sampling time point. The fast excretion of [^{3}H]-DON (above the average gastric emptying time of trout) might be related to its high water solubility and consequent excretion with the fluid phase of the chyme. The amount of [^{3}H]-DON in the GIT was stable during the first six hours. Such long transit time of DON through the GIT increases the potential for damage and absorption. The period between six and twelve hours seems to be the turning point in terms of DON excretion. Twelve hours after tube-feeding, the trout excreted 50.71 ± 22.17% of the tube-fed DON amount into water, while at the previous sampling time point (six hours) only 11.03 ± 6.09% were detected. These data suggest that an effective method for gastrointestinal DON detoxification in trout requires a period of action lower than six hours. In the present trial, no DON metabolites were detected in water.

Keywords: *Fusarium* mycotoxin; toxicokinetics; deoxynivalenol (DON); tube-feeding

4.1 Introduction

Fusarium mycotoxins are the most prevalent mycotoxins found in aquafeeds, reflecting the type and inclusion levels of plant meals used in these diets (Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). Moreover, the presence of secondary metabolites of *Fusarium* spp. are expected to increase in aquaculture raw materials in response to climate change (Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011), which might represent a challenge for the aquaculture industry. Among the metabolites produced by the genus *Fusarium*, deoxynivalenol (DON) is reported to be the main mycotoxin found in small grain cereals (Rodrigues and Naehrer, 2012; Simsek *et al.*, 2013). Absorption, distribution, metabolism and elimination (ADME) of DON differs among animal species (Pestka, 2007). The toxic effects and toxicokinetics of DON are well described for land farmed animals (Pestka, 2007), but less is known for aquatic animals. Only recently, Bernhoft *et al.* (2017) evaluated the tissue distribution and elimination of DON in Atlantic salmon (*Salmo salar*), considering also the possibility of accumulation of mycotoxins or their metabolites in fish tissues. Bernhoft *et al.* (2017) reported that DON was present in liver, kidney, muscle, skin and brain of Atlantic salmon after treatment with 6 mg kg^{-1} DON for eight weeks.

For aquatic animals, almost nothing is known about the metabolism of DON. However, for terrestrial animals, it was observed that DON can induce phase I and II liver biotransformation enzymes (Gouze *et al.*, 2005). Advancements in the knowledge of DON toxicokinetics and metabolism in fish will support risk assessment of DON for aquatic species and its counteraction.

Taking into account that little is known about the fate of DON in fish, especially with regard to excretion and biotransformation, the objective of the present study was to evaluate the pharmacokinetic behaviour of radio-labelled DON ([^{3}H]-DON) in rainbow trout (*Oncorhynchus mykiss)*, focusing on tissue distribution, excretion and possible DON biotransformation.

4.2 Materials and methods

4.2.1 Ethics statement on animal experiments

All experimental procedures involving animals followed the EU Directive 2010/63/EU and National *Decreto-Lei 113/2013* legislation for animal experimentation and welfare.

Animal handling and experiments were performed by qualified operators accredited by the Portuguese *Direção-Geral de Alimentação e Veterinária* (DGAV). This study was conducted at the Center for Marine Sciences (CCMAR) of Universidade do Algarve, Faro, Portugal.

4.2.2 Husbandry and fish nutritional background

Juvenile rainbow trout (*Oncorhynchus mykiss*) with a mean initial body weight of 7.72 ± 1.42 g were acclimatised in 40 L cylinder-conical fiberglass tanks over the course of three weeks. During the acclimatisation period, fish were fed a mycotoxin-free diet at an amount corresponding to 1.5% body weight, four times a day via automatic feeders. Fish were kept at a density of less than 2 kg m^{-3}, in a recirculation freshwater system at 15 ± 1.0 °C, with a 12 h Light: 12 h Dark photoperiod. Dissolved oxygen levels were kept above 90% oxygen saturation.

4.2.3 Pellets labelled with [^{3}H]-deoxynivalenol

For the metabolic trial, each feed pellet was individually labelled with 31.25 ng of the tracer, [^{3}H]-DON (3.7 MBq; American Radiolabeled Chemicals Inc., The Netherlands). The tracer was directly added in the dry pellet with a pipette and leave it to be absorbed. After labelling, the pellets were dried at 50 °C for 30 minutes and stored at 8 °C for the subsequent tube-feeding procedure.

4.2.4 Experimental procedure

After the acclimatisation period, rainbow trout juveniles were tube-fed with pellets containing radiolabelled DON. Tube-feeding was performed according to the method described by Rust *et al.* (1993), modified by Costas (2011). Randomly selected fish (n = 6, for each sampling time point) were transferred to the laboratory after being fasted for 18 h. In brief, fish were anesthetised (ethyl 3-aminobenzoate - MS-222, Sigma) and tube-fed with four pellets of the diet each (corresponding to 0.13% body weight and a total of 125 ± 0.019 ng of [^{3}H]-DON). For tube-feeding, a hollow plastic tube of 1.5 mm inner diameter and a solid piece with a smaller diameter placed inside as a plunger were used. The diameter and length of the plastic tubing was previously tested to avoid injuring the oesophagus of the rainbow trout juveniles. Tube-fed fish were allowed to recover for 10 minutes in clean, fresh water to eliminate any residual anaesthetic from

the skin and gills, and monitored for possible pellet regurgitation. After this period, fish were transferred to the incubation chamber (individually housed) tempered to 15 ºC. Each chamber (2 litres) was hermetically sealed, and supplied with a gentle oxygen flow. After the incubation period (1 h, 3 h, 6 h, 12 h or 24 h; 6 incubation chambers), oxygen flow was stopped and fish were sacrificed inside the chambers using a lethal dose of the anaesthetic (ethyl 3-aminobenzoate - MS-222, Sigma).

After fish removal, water from each incubation chamber was collected (5 mL aliquots per chamber) for radioactive counting to infer the quantity of mycotoxin excreted by the fish. Fish were individually weighed and sampled for muscle (without skin), skin, liver, kidney and gastro-intestinal tract (GIT). All samples were weighed, except those from the kidneys due to its low weights and the absence of accurate scales in the radioactivity laboratory. Muscle and skin samples were not collected from fish incubated for one hour, as digestion is assumed to take longer than 1 hour, and therefore, radioactivity was not be expected to reach these tissues. Blood was not collected due to the small size of the fish and coagulation of the blood after the anaesthetic overdose.

4.2.5 Mycotoxin fate determination

Samples of the dissected tissues were completely dissolved in Solvable^{TM} (Perkin Elmer, USA) at 50 °C for 24 h. Radioactivity in tissue samples (muscle, skin, liver, GIT), water samples and pellets (n = 50; to confirm labelling success) were quantified by scintillation counting in a Tri-Carb 2910TR low activity liquid scintillation analyser (Perkin Elmer, USA) after addition of Ultima Gold XR scintillation cocktail (Perkin Elmer, USA). The metabolic budgets were calculated after subtraction of blanks for quench and lumex correction.

4.2.6 Deoxynivalenol metabolism assay

For the DON metabolism study, each pellet was supplemented with 165.75 ng of unlabelled DON, giving a total dose of 663 ng of DON per fish (four pellets per tube-fed animal). After adding DON, the pellets were dried at 50 °C for 30 minutes and then left to cool at room temperature before the subsequent tube-feeding procedure. The experimental procedure was performed as for the [^{3}H]-DON treatment, with the only difference being that water was the only sample collected from the metabolic chambers, after 24 h of incubation, in order to identify and quantify excreted DON and its potential

metabolites. No fish tissues were analysed during this assay. Water collected from the chamber (50 mL aliquots from each chamber), was analysed by LC-MS/MS for the presence of DON and potential DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) as described by Streit *et al.* (2013).

4.2.7 Determination of toxicokinetic parameters

Toxicokinetic parameters were determined for GIT and liver collected during the [^{3}H]-DON experiment. All calculations were based on the assumption that 1 DPM equals 0.22 pg of mycotoxin. DON concentration at zero time (C_0) was determined from the tissue concentration–time curves obtained. Elimination constants (K_{el}) were determined by curve regression ($C(t)=c_0*e^{(Kel*t)}$). The elimination half-life, the time necessary to halve the concentration, was calculated as in $t_{1/2}=\ln 2/K_{el}$. C_{max} is the peak of [^{3}H]-DON concentration in the respective tissue at a certain time (t_{max}). Toxicokinetic parameters were not determined for skin and muscle due to the insufficient sampling points.

4.2.8 Statistical analysis

Results are presented as means ± standard deviation (S.D.). Results expressed as percentage were arcsine-transformed prior to statistical analysis. Statistical analyses were performed using the STATISTICA version 8.0 software (StatSoft Inc.). Data were verified for normal distribution and homogeneity of variances. Significant differences between groups (samples taken at the same time point) were assessed by one-way ANOVA. When significant differences were detected, the Tukey's multiple-comparison test was used to assess differences between groups. Differences were considered to be significant when $p < 0.05$.

4.3 Results

4.3.1 Deoxynivalenol distribution and excretion

Pellets presented a mean value of 151,282 disintegrations per minute (DPM). Results for mycotoxin fate in rainbow trout are expressed based on the assumption that 1 DPM equals 0.22 pg of DON.

The DON distribution in fish tissue (ng of DON; GIT, liver, kidney, muscle, kidney and skin) and in water, for each sampling point, and the percentage of DON in tissues (sum

of tissues per sampling point) or in water relatively to the tube-fed amount (125 ± 0.019 ng DON) is shown in table 4.1. One hour after tube-feeding, [^{3}H]-DON was detected mainly in the GIT (20.56 ± 8.30 ng), and low levels were detected in the liver (1.44 ± 0.67 ng) and kidneys (0.23 ± 0.13 ng) (muscle and skin were not sampled at one hour post tube-feeding). At one hour sampling time point, 6.19 ± 0.83 ng [^{3}H]-DON was detected in the water (Table 4.1). At this sampling point [^{3}H]-DON in water represented 4.94 ± 0.66 % of the ingested [^{3}H]-DON, being statistically lower than the percentage found in the tissues (17.74 ± 6.71, $p = 0.001$). No differences ($p > 0.05$) were observed during the 24 h period regarding the presence of [^{3}H]-DON in kidney, liver and skin of the trout juveniles, which remained relatively low. GIT showed a relatively constant amount of [^{3}H]-DON during the first six hours, decreasing after this to a final amount of 10.02 ± 10.45 ng DON at twenty-four hour. No statistical differences were found for this tissue for the twenty-four hours experimental period.

After twelve and twenty-four hours, the percentage of DON present in water (63.50 ± 27.76% and 62.15 ± 35.56%, respectively) compared with the total tube-fed DON (125±0.019 ng DON), was significantly higher ($p = 0.001$) than in previous sampling points (1 h = 4.94 ± 0.66, 3 h = 16.62 ± 12.80 and 6 h = 11.03 ± 6.09%). The total recovery of [^{3}H]-DON also increased significantly at twelve and twenty-four hours sampling (69.86 and 75.22%, respectively).

At the end of the experimental period (24 h of being tube-fed) trout presented marginal amounts of [^{3}H]-DON in the tissues (GIT = 10.02 ± 10.45 ng; liver = 0.87 ± 0.85 ng; muscle = 3.61 ± 2.84 ng; kidney = 0.57 ± 0.46 ng and skin = 1.58 ± 1.57 ng). At this time, most of the [^{3}H]-DON was found in the water (77.84 ± 44.54 ng), representing 62.15 ± 35.56% of the initial tube-fed DON (125 ± 0.019 ng DON). The period between six and twelve hours after tube-feeding seems to be the turning point where it is possible to observe a higher level of [^{3}H]-DON being excreted into the water compared to levels of [^{3}H]-DON in the fish (Figure 4.1). After twenty-four hours, the sum of [^{3}H]-DON in all tissue samples showed a total of 16.37 ± 14.46 ng of ^{3}H-DON compared to 77.84 ± 44.54 ng ^{3}H-DON excreted into the water (Figure 4.1).

Table 4.1: Deoxynivalenol (DON distribution in fish tissue (ng of DON; gastro-intestinal tract, liver, kidney, muscle, kidney and skin) and in water, after tube-feeding pellets labelled with [^{3}H]-DON, for each sampling point (one to 24 hours). In addition, percentage of DON in tissues (sum of tissues per sampling point) or in water relatively to tube-fed amount (125 ± 0.019 ng DON).

	Sampling points									
	1H		**3H**		**6H**		**12H**		**24H**	
Water/ tissues	**DON (ng)**	**% of DON relative to ingested DON**	**DON (ng)**	**% of DON relative to ingested DON**	**DON (ng)**	**% of DON relative to ingested DON**	**DON (ng)**	**% of DON relative to ingested DON**	**DON (ng)**	**% of DON relative to ingested DON**
Water	6.19±0.83^{c}	4.94±0.66^{a}	20.81±16.04^{bc}	16.62±12.80^{a}	16.58±3.93^{c}	11.03±6.09^{a}	63.50±27.76^{b}	50.71±22.17^{b}	77.84±44.54^{a}	62.15±35.56^{b}
GIT	20.56±8.30		18.27±1.88		21.79±7.95		13.14±6.14		10.02±10.45	
Liver	1.44±0.67		0.93±0.17		1.24±0.54		1.14±0.55		0.87±0.85	
Muscle	n.s.	17.74±6.71^{b}	6.14±2.48^{ab}	22.30±3.48^{b}	8.89±4.00^{a}	28.80±8.54^{b}	6.67±2.30^{ab}	19.15±7.70^{a}	3.61±2.84^{b}	13.07±11.54^{a}
Kidney	0.23±0.13		0.30±0.20		0.52±0.41		0.31±0.08		0.57±0.46	
Skin	n.s.		2.44±1.35		3.62±2.30		2.78±1.14		1.58±1.57	
[^{3}H]-DON Recovery	-	22.68%	-	38.92%	-	39.83%	-	69.86%	-	75.22%

Values are means ± S.D. for each sampled tissue and water. Different letters indicate statistically significant differences ($p < 0.05$, one-way ANOVA) between time points for tissues and water. Kidney, liver, GIT and skin did not show significate differences during the 24-hour period. For the percentage of DON relative to ingested DON different letters indicate statistically significant differences ($p < 0.05$, one-way ANOVA) within same sampling time point between water and sum of tissue. n.s. = not sampled.

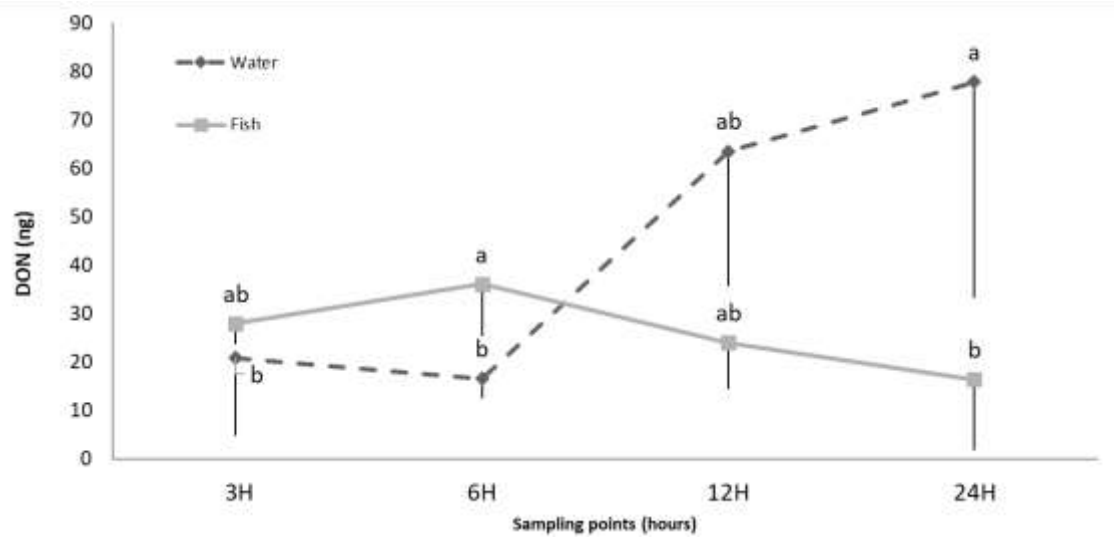

Figure 4.1: Deoxynivalenol (DON) in fish tissue (sum of total DON detected in the gastro-intestinal tract, liver, kidney, muscle and skin) and excretion into water at 3 h, 6 h, 12 h and 24 h after tube-feeding a meal labelled with ^{3}H-DON. Values are means ± S.D. Different letters within each compartment indicate statistically significant differences ($p < 0.05$, one-way ANOVA) between sampling points.

4.3.2 Determination of toxicokinetic parameters

The toxicokinetic parameters of [^{3}H]-DON in tube-fed rainbow trout are presented in Table 4.2. The distribution and excretion profiles differed depending on the tissue. GIT presented the highest concentration of [^{3}H]-DON (C_{max} = 65.28 ng g $^{-1}$) after 3 h of tube feeding (t_{max} = 3 h). This maximum concentration decreased thereafter with a half-life ($t_{1/2}$) of 88.51 h (four time points considered, r^2 = 0.706). Samples from the liver showed a lower peak concentration of [^{3}H]-DON (C_{max} = 12.91 ng g $^{-1}$), also at 3 h after tube feeding (t_{max} = 3 h) however, with a $t_{1/2}$ of 95.14 h (four time points considered, r^2 = 0.444). Toxicokinetics for kidney, muscle and skin were not calculated.

Table 4.2: Toxicokinetic parameters of [^{3}H]-DON tube-fed to rainbow trout

	GIT	**Liver**
Interception	1.80	1.08
Slop	-0.003	-0.003
C_{max}	65.28	12.91
t_{max}	3	3
C_o	63.08	12.26
K_{el}	0.008	0.007

$t_{1/2}$	88.51	95.14

Concentration at time zero (C_0; ng g^{-1} DON) was determined from the tissue concentration–time curves obtained. Elimination constants (K_{el}; h) were determined by curve regression ($C(t)=c0*e(K_{el}*t)$). The elimination half-life was calculated as in $t_{1/2}=\ln 2/K_{el}$. C_{max} (ng g^{-1} DON) is the peak concentration of [^{3}H]-DON after administration at a certain time (t_{max}, h). Toxicokinetics for muscle and skin were not calculated.

4.3.3 Deoxynivalenol metabolism assay

We did not detect any DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) in the water samples taken after 24 h from the chambers of the tube-fed animals that received 663 ng of DON each (limits of detection: 0.2 µg kg^{-1} for DOM-1, DON-3-sulfate and DOM-3-sulfate)., We detected 0.08 ± 0.063 ng of DON per ml of water . The recovery of DON represents 24.13% of the total amount fed via a tube to the animal.

4.4 Discussion

Understanding the biological fate of DON in aquaculture species is of vital importance, as it sheds light on the carryover of DON into edible tissues and on possible adverse effects of the toxin on the animal. Knowledge of the biological fate of DON also provides understanding of how to address and mitigate the impact of DON in the animal, for example by developing a DON-detoxifying feed additive. Detoxifying feed additives are intended to decrease the bioavailability of the toxin to the animal in the digestive tract, reducing any possible negative effect of the toxin on the GIT and its absorption into the blood. Knowledge about retention time of DON in digesta, absorption, and distribution, as well as possible biodegradation of DON by the indigenous GIT bacteria are fundamental to the development of a DON-detoxifying strategy. In the current trial, [^{3}H]-DON was detected in all sampled tissues (GIT, liver, muscle, kidney and skin). One hour after tube-feeding, [^{3}H]-DON was detected in the liver of the fish, indicating that DON absorption is relatively fast in rainbow trout juveniles. Due to technical challenges, it was not possible to collect plasma for analysis. Analysis of DON levels in plasma could have confirmed that DON was absorbed quickly. Bernhoft *et al.* (2017) reported that the concentration of DON found in *Salmo salar* liver samples reached a maximum concentration one hour after intake. In the present study for rainbow trout, the maximum concentration was only achieved after 3 h

and half-life was higher ($t_{1/2,liver}$ = 95.14 h) than reported by Bernhoft *et al.*, 2017 ($t_{1/2,liver}$ = 6 h), which might explain the higher sensitivity of trout to DON (Hooft *et al.*, 2011). Bernhoft *et al.* (2017) obtained a maximum DON plasma concentration at time zero and $t_{1/2\ plasma}$ = 15.1 h, showing that DON is rapidly absorbed in salmon. Moreover, the low $t_{1/2\ plasma}$ reported by Bernhoft *et al.* (2017) indicates the possibility of some absorption of DON from the stomach. A rapid absorption of DON was also observed by Dänicke *et al.* (2004) and Eriksen *et al.* (2003) in pigs.

Focusing on the first hour after tube-feeding, [^{3}H]-DON was detected mainly in the GIT (20.56 ± 8.30 ng). However, some radioactivity was also detected in the water (equivalent to 6.19 ± 0.83 ng [^{3}H]-DON). As regurgitation can be excluded (visual confirmation), any [^{3}H]-DON detected in the water was excreted and not vomited or leached from the pellets. The low passage time of [^{3}H]-DON through the GIT (< 1 hour), which was lower than the trout average gastric emptying time (> 6 h; depending on temperature and meal type and size; see Langton (1977)), could be due to the high water solubility of DON and excretion of DON with the fluid phase of the chyme. Accordingly, Dänicke *et al.* (2004) reported that in pigs, DON leached from pellets into the liquid phase in the stomach and was emptied with the liquid phase of the chime and faster than the solid phase of the chyme. While the rapid excretion of DON may prevent immediate negative effects of dietary DON on the GIT of the trout, the high solubility and stability of DON in water may lead to re-ingestion by the fish.

The tube-feeding technique was selected to simulate a normal pellet intake, eliminating the risks of DON leaching from the pellets and ensuring the intake of a defined amount of DON. While the employed experimental setup revealed the rapid passage of [^{3}H]-DON through the GIT, which is an important and novel information, it was associated to some technical challenges in the methodology used. The recovery of [^{3}H]-DON, especially during the first three sampling time points (1, 3 and 6 hours) was relatively low (22.68%, 38.92% and 39.83%, respectively). Despite the metabolic chambers being a closed system, some losses were expected due to sampling limitations. For instance, DON residues in the head and skeleton were not analysed and DON residues in the blood could not be analysed due to coagulation during anaesthetic overdose euthanasia. This inevitably contributed to losses in the [^{3}H]-DON budget. Indeed, *Salmo salar* brain was shown to absorb DON (Bernhoft *et al.*, 2017). Arguably, the most important factor that contributed to the low recovery of [^{3}H]-DON was the loss of material from the GIT during sampling. While most of the solid phase of the chyme and the faeces remained in

the GIT during tissues sampling, the fluid phase of the chyme was probably lost during the sampling procedure. Consequently, a loss of DON contained in the fluid phase may have contributed to the recovery of constantly low [^{3}H]-DON levels from the GIT at the first sampling time points (1 to 6 h). At twelve and twenty-four hours, [^{3}H]-DON recovery was higher, namely 69.86% and 75.22% respectively. At these time points recovery was probably mostly influenced by tissues not collected (head, blood, skeleton with muscle attached) as digestion had already happened.

The low absorption of DON during the first 1, 3 and 6 hours (17.74 ± 6.71; 22.30 ± 3.48; 28.80 ± 8.54% in relation to tube-fed DON amount, respectively) may also be explained by the trout's physiological condition prior to the study. In the present trial, trout were fed a non-contaminated diet (during three weeks) prior to the [^{3}H]-DON tube feeding. It has been reported that chronic exposure to DON might cause the destruction of tight junctions (Mayer *et al.*, 2017; Pinton *et al.*, 2012) leading to increased DON absorption. We assume that in the present study, the trout's physiological conditions due to the three weeks acclimation were optimal and intestinal barrier would not be much impacted by the short period of DON exposition (maximum of twenty-four hours).

For future studies, it would be interesting to adapt the experimental procedures for the tube-feeding technique in order to enable us to collect the fluid phase of the chyme from the GIT during sampling and to determine DON residues in the fluid phase, especially during the first six hours of sampling (maximum expected digestion time). Furthermore, due to a possible harmful effect of chronic DON exposure on the intestinal barrier it would be relevant to assess the toxicokinetics of DON in trout chronically exposed to DON before the toxicokinetic experiment. The low excretion of DON into the water during the first six hours after DON tube-feeding indicates a long DON retention time that increases the probability of DON absorption and of a negative effect of DON on the GIT The period between six and twelve hours seems to be the turning point in terms of DON excretion. Twelve hours after tube-feeding, the trout excreted 50.71 ± 22.17% of the tube-fed DON amount, while on the previous sampling point (six hours) the trout excreted only 11.03 ± 6.09%. Taking into account a digestion time of six hours at 15 ^{o}C (Langton, 1977) it can be expected that most of the DON was retained in the GIT and excreted after digestion.

In a previous study, our group found that DON is metabolised to DON-3-sulfate in trout (Gonçalves *et al.*, 2018e). In this previous study, more than 80% of the mycotoxin

recovered from faeces was DON-3-sulfate. The location of the formation, absorption and elimination of DON-3-sulfate has not been identified, but evidence suggested that DON might be metabolised into DON-3-sulfate in the intestinal mucosa (Schwartz-Zimmermann *et al.*, 2015). In the present trial, only DON was found in the water from metabolic chambers and no DON-3-sulfate was detected. Based on this discrepancy, it is tempting to speculate that the conversion of DON to DON-3-sulfate is catalysed by the gut microbiota and that its incidence depends on gut microbial community composition. As there were no DON metabolites detected, the detected radioactivity likely originated from intact DON molecules. However, unknown DON metabolites may have been missed. The low concentrations of DON measured in this trial were near the limit of detection of the analysis method (0.2 µg kg $^{-1}$). This might have contributed to the low recovery of non-radiolabelled DON.

4.5 Conclusions

Despite some limitations of the experimental procedures, which influenced the obtained results, especially during the first three sampling points, we could conclude that one hour after tube-feeding, [^{3}H]-DON was detected in the liver samples of fish, indicating a rapid absorption of DON. In the first hour, [^{3}H]-DON was present in the GIT (20.56 ± 8.30 ng). However, 6.19 ± 0.83 ng was also detected in the water at this sampling point. The fast excretion of [^{3}H]-DON (faster than the average trout gastric emptying time) suggests that DON, as a water-soluble compound, is excreted with the liquid phase of the chyme. The presence of [^{3}H]-DON in the GIT was stable during the first six hours. This long residence time of DON in the GIT may compromise the health of the GIT and favour absorption. Our data suggests that an effective DON detoxifying method should have a period of action of ≤ 6 h. Furthermore, as most of the excretion can be expected to happen after six hours, the detoxification should be irreversible at GIT conditions.

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CHAPTER 5

General discussion

5.1 Industry awareness about mycotoxins in aquafeeds

Traditionally, the inclusion of minor levels of plant feedstuffs in aquafeeds, led to a general perception that mycotoxins were not a relevant issue in aquaculture and that the majority of mycotoxin issues would stemmed only due to poor storage conditions (*Aspergillus* spp. and *Penicillium* spp. contamination). This would be particularly true for some countries where climate conditions are favourable to the growth of *Aspergillus* spp. and *Penicillium* spp. fungi. However, even in these cases, optimal storage conditions should prevent the contamination of raw materials and finished feeds from AF or OTA. Moreover, some plant commodities such as cottonseed and peanut meals are commonly contaminated with high levels of AF and/or OTA (Gonçalves *et al.*, 2017). Meaning that the use of such raw materials, typical AF/ OTA contaminated, might represent a camouflage threat, even at optimal storage conditions and with no visible fungi contamination. It is also believed, for example, that aflatoxin as the main contamination in aquafeeds, is strictly seasonal or restricted to certain raw materials, which is not correct as shown in **Chapter 1**. These incorrect concepts can lead aquaculture companies to implement wrong mycotoxin management strategies, leading to a false sense of security, which might lead to economic losses due to diseases and feed inefficiency losses, being these losses hard to explain and rarely attributed to mycotoxins due to a lack of specific clinical symptoms in aquaculture species.

Fusarium mycotoxins (Type B and A trichothecenes and Fumonisins) are being pointed as the main mycotoxins found in shrimp and fish feeds (Gonçalves *et al.*, 2018b; Gonçalves *et al.*, 2018d; Gonçalves *et al.*, 2017). The lack of studies reporting *Fusarium* mycotoxins contamination in periods earlier than 2012, seems to be related to the lack of interest/ awareness about other mycotoxins besides AF's rather than the limited access to mycotoxin analytical methods for these other mycotoxins. On one hand, this does not necessarily mean that *Fusarium* spp. contamination was lower in the past and on the other hand, may have been supported the generalized wrong concept that aflatoxins were the most predominant mycotoxins in aquafeeds.

Surveying the mycotoxin risk of plant meals to be used in aquafeeds is probably the best option on which to base management of any possible risk coming from these feedstuffs. As it is not possible to assume that a certain commodity will always have the same mycotoxin contamination pattern or that contamination will be within a certain contamination range. Fusarium mycotoxins (Type B and A, trichothecenes and fumonisins) are, contrary to AF and OTA, mainly produced at the pre-harvest stage. The production of these mycotoxins by *Fusarium* spp. seems to be highly influenced by environmental conditions, so an increase in occurrence is expected due to climate change (Miraglia *et al.*, 2009; Paterson and Lima, 2010; Paterson and Lima, 2011).

5.2 Consequences of mycotoxins in aquaculture species: myth or reality?

The awareness of mycotoxin-related issues in the aquaculture industry has been clearly increasing, accentuated by the increased inclusion levels of plant meals in aquafeeds and this is obvious by the amount of recent literature being published in this topic (Tables S1.4 to S1.12). However, despite the existing literature on the topic is still difficult to draw firm conclusions about sensitive levels for aquatic species. This because, in some cases, published literature tends to test unrealistically high levels of mycotoxins when compared to the mycotoxin contamination level found in aquaculture feeds (Boonyaratpalin *et al.*, 2001; Manning *et al.*, 2014; Tuan *et al.*, 2003). Testing unrealistic high values might suggest a dangerous message to the aquaculture industry. Firstly, this tends to pass the wrong idea that mycotoxin occurrence in aquafeeds is relatively low and therefore safe. Secondly, readers tend to expect the same clinical signs obtained in these studies, with high levels of mycotoxin contamination, which might not happen for lower contamination levels and again lead to an unrealistic sensation of security. Moreover, the high number of aquaculture species and factors such as exposure period (addressed in **Chapter 2**) age, nutritional and health status, rearing densities and environmental conditions, might influence the outcome of trials, leading to variable sensitivity levels, sometimes even for the same species. Additionally, there are no studies addressing the possible synergism of mycotoxins. Therefore, is highly recommended that scientist, when selecting the mycotoxin experimental levels, would follow EU guidance values for the chosen mycotoxin and experimental model. In case of not existing such information would be advised to choose testing levels according to livestock reference values.

However, the biggest barrier to quantify the impact of mycotoxin contamination in the aquaculture species is the apparent lack of clinical signs or biomarkers for aquatic species mycotoxin exposure, especially when compared to terrestrial livestock. Several reports describe broad and non-specific clinical signs for the most common mycotoxins (see a review from Anater *et al*. (2016)). However, this lack of specific clinical signs might attribute mycotoxin-related problems to other pathologies or challenges (e.g., *Yersinia ruckerri*, **Chapter 2**). The case of aflatoxicosis, (yellowing of the body surface, (Deng *et al.*, 2010) and ingestion of fumonisins (FUM; alteration of the sphinganine to sphingosine ratio, (Tuan *et al.*, 2003) are two notable exceptions, however, as mentioned before is not yet validated that these clinical signs may be extrapolated to other species and to which extent these clinical signs are dosage/ exposure-dependent. Also, during this thesis (**Chapter 3**; Gonçalves et al. (Gonçalves *et al.*, 2018e)) described DON-3-sulfate as a potential biomarker of deoxynivalenol (DON) exposure in rainbow trout (*Oncorhynchus mykiss*). Therefore, finding parameters (biomarkers or specific clinical signs) which would help aquaculture industry to clearly identify mycotoxicoses is a top priority in this research field and which to a certain extent, this thesis successfully contributed.

5.3 Trout's deoxynivalenol exposure

5.3.1 Effect of contamination level and exposure period

One of the main constraints when researching mycotoxins in aquaculture species is the lack of mycotoxin-induced clinical symptoms. Two of the factors that highly influence the manifestation of clinical signs are the exposure period and mycotoxin contamination level. In **Chapter 2**, is described as the effects of DON contamination in trout, exploring two different scenarios. In the first scenario, the effect of short exposure period of high levels of DON (50 days; 1,166 µg kg $^{-1}$ DON and 2,745 µg kg $^{-1}$ DON) and in a second scenario, the evaluation of the effects of long exposure period to low levels of DON (168 days; 367 µg kg $^{-1}$ DON). In the short-term/high DON exposure experiment, 15 out of 60 fish that were fed 2,745 ± 330 µg kg $^{-1}$ DON showed an abnormal body conformation, characterized by a fish length reduced in relation to its width, and five out of 60 fish from same treatment presented a protruding anal papilla. While no clinical signs were observed after long-term exposure/low DON contamination. Regarding the fish fed, the high DON dosage during short-term

exposure, clinical manifestation was only observed in a small number of individuals, which indicates that there is a certain inter-individuality variability response to DON ingestion. With the work presented in **Chapter 2**, we concluded that, at least in rainbow trout and probably in other aquaculture species, the clinical manifestation of DON ingestion are dosage and exposure period dependent. This also highlights the fact that clinical manifestations at higher contamination levels might not be extrapolated to lower contamination levels, even in the same species. Actually, in the experiment described in **Chapter 3**, (Gonçalves *et al.*, 2018e) no macroscopic lesions were found (i.e., internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) on rainbow trout that were fed high levels of DON during a short exposure period (11,412 ± 1,141 µg kg $^{-1}$; 60 days). Another two other studies with the same range of DON contamination (0.3 to 5.9 µg kg $^{-1}$), Hooft *et al*. (2011) and Ryerse *et al*. (2015) also reported no major pathological changes in the distal intestine of trout, while Matejova *et al*. (2014) reported gastrointestinal haemorrhages, reinforcing the highly variable response to DON ingestion in rainbow trout.

Remaining altered parameters obtained in the experiment described in **Chapter 2**, i.e., reduction in feed intake and general growth performance indicators (SGR, FCR, TGC and PER) are well-documented responses of trout to diets contaminated with DON at high levels during short exposure periods (Gonçalves *et al.*, 2018c; Hooft *et al.*, 2011; Ryerse *et al.*, 2015). Long-term exposure to DON is described for the very first time at the present thesis and was observed that ingestion of DON is asymptomatic, as the animals presented no clear/specific clinical signs, being growth rate only affected after 92 days of ingesting DON. In both DON exposure periods experiments, it was difficult to correctly diagnose DON intake using liver enzymes and histology. In the short-term/high DON exposure study, histological and enzymatic changes showed high individual variability. Moreover, macroscopically lesions, e.g., protruding anal papilla, also showed high variability. The high variability of clinical signs observed in the animals fed DON, makes difficult to correctly diagnose DON ingestion. The possible causes for such variability will be further addressed in **sub-chapter 5.3.2**.

The intake of DON has been also reported to lead to the upregulation of cytokine levels, especially pro-inflammatory cytokines (*IL-6*, *IL-8* and *IL-1β*). In several studies (piglets, (Bracarense *et al.*, 2012)); human intestinal Caco-2 cells (Maresca *et al.*, 2008; Van De Walle *et al.*, 2008); and mice ((Azcona-Olivera *et al.*, 1995)). Intestinal upregulation of

pro-inflammatory cytokines was not measured in the experiment reported in **Chapter 2**; however, it may explain the higher resistance of DON-treated fish to infection with *Y. ruckeri*. The higher resistance to pathogen infection may be considered, at first sight, as a positive effect, however, the reduced feed intake and lower growth performance may have economic consequences for aquaculture.

When comparing with the clinical signs of DON ingestion described for livestock species (Pestka, 2007; Rotter *et al.*, 1996), DON toxicity includes reduced growth and feed intake (also common to aquaculture species), vomiting and diarrhoea (not observed in aquaculture), gastrointestinal haemorrhaging, inflammation and alteration of the immune response (observed in aquaculture depending on DON exposure level and period). Actually, it is curious that the rectal prolapse observed in some trout's (**Chapter 2**) are also typical clinical manifestation described in swine when fed 3,000 µg kg $^{-1}$ DON (Madson *et al.*, 2014).

The high levels of individual variability observed in the blood biochemical parameters, histological changes and clinical signs in the fish that were fed high levels of DON during short periods and the lack of symptoms in trout's fed low levels of DON chronically, despite the reduction in growth performance are further addressed in **sub-chapter 5.3.2**. The experiment described in **Chapter 3**, attempts to understand the, so far, unexplainable variability in clinical manifestation upon DON ingestion, which might be, within others, related to the DON biotransformation achieved by the gut microbiota and will be further discussed in next sub-chapter.

5.3.2 Depict the growth performance decrease and lack of clinical signs

5.3.2.1 Digestibility and enzymatic activity

Chapter 3 explores the underpinning causes for the reported decreases in growth performance and feed intake previously described in **Chapter 2**. Despite the higher DON concentrations tested (4,714 ± 566 and 11,412 ± 1,141 µg kg $^{-1}$), growth performance was affected by DON, in a similar manner to that previously described in **Chapter 2** and as in literature (Gonçalves *et al.*, 2018c; Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Matejova *et al.*, 2015; Ryerse *et al.*, 2015). However, contrary to previous experiment (chapter 2; (Gonçalves *et al.*, 2018c)), no macroscopic lesions (i.e., internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) were detected on animals fed DON. In addition to the

lower growth in the DON fed treatments, which was visually detected (Figure 3.1), fish fed 11,412 ± 1,141 µg kg $^{-1}$ DON also had significantly lower survival rates. This lack of clinical signs, even at extremely high levels of DON contamination, as well the high individual variability of the affected parameters, makes it highly unlikely that under production conditions, such increase in mortality/decrease in performance would be associated to DON ingestion.

The decrease of enzymatic activity, or/ and to the formation of complexes with proteins thereby modifying digestion processes is well described to some anti-nutritional factors (Moyano *et al.*, 1999; Santigosa *et al.*, 2008) and this may be the case of DON. Trout's fed high levels of DON saw its proteolytic enzyme activity (pepsin, trypsin and chymotrypsin) significantly altered, which may have contributed to the lower efficient protein turnover, explaining like this the lower growth performance. Trypsin may be one of the key factors explaining the lower protein turnover efficiency, which showed lower activity in DON fed groups. Also interesting is the observation that from the four types of alkaline proteases (*ctrl*; *try1*, *try2* and *try3*), only mesotrypsinogen (trypsinogen-3) showed a higher mRNA expression level in DON fed treatments. Mesotrypsin is a specialised protease known for its resistance to trypsin inhibitors. It is thought to play a special role in the degradation of trypsin inhibitors, possibly to help with the digestion of inhibitor-rich plant meals such as soybeans and lima beans (Szmola *et al.*, 2003), which might be the case of plant meals containing DON as well, however, more research is needed on this topic.

Following up a justification for the lower protein turnover efficiency, both trypsin activity and mRNA expression levels for *igf1* and *igf2*, were found to be significantly higher in the CTRL when compared to DON fed groups. It is well known that trypsin cleaves protein at the carboxyl side of the basic amino acids lysine and arginine (Stryer, 1988), which elevates plasma insulin levels in salmonids (Plisetskaya *et al.*, 1991). In turn, insulin stimulates amino acid uptake and protein synthesis especially in the muscle tissue (Matty, 1986; Murat *et al.*, 1981), leading to growth promoting effect in salmonids (Donaldson *et al.*, 1979). Proteolytic enzyme differences observed between the treatments were probably not associated with secretion control, as cholecystokinin-like peptides (*cck-t*; *cck-n* or *cck-l*) did not significantly alter due to DON treatment. DON influence on trypsin activity, and the consequent possible trypsin influence in the levels of insulin may ultimately influence amino acid uptake, which will ultimately

explain the lower protein retention and therefore lower growth in animals fed DON. However, this requires further research to verify whether DON directly affects this pathway. Furthermore, the fact that nutrient retention (protein, fat and energy) were significantly affected in animals fed DON, the low performance of the animals fed DON could be a consequence of decreased nutrient uptake and transport rather than lower nutrient digestibility, as lipase and amylase enzyme activity and ADC appear unaffected by DON.

5.3.2.2 Appetite regulation

The neuroendocrine process that controls satiety is regulated, with others, by Neuropeptide Y, Leptin, Ghrelin or Adenylate cyclase-activating polypeptide (PACAP). Leptin and ghrelin mRNA transcripts were not influenced by DON (**Chapter 3**). This was not surprising as Leptin activity is related to long-term regulation of energy balance, suppressing food intake, while Ghrelin is a fast-acting hormone acting as "stopper" after meal initiation (Klok *et al.*, 2007). In contrast, up-regulation of *adcyap1a* or PACAP mRNAs observed in **Chapter 3**, provides a possible link to the observed reduction in feed intake, as described in the literature (Chance *et al.*, 1995; Li *et al.*, 2015; Morley *et al.*, 1992; Mounien *et al.*, 2008; Tachibana *et al.*, 2003). PACAP plays an important and direct role in the regulation of feed intake in fish. In goldfish, it has been observed that intracerebroventricular injections of PACAP suppress food intake (Matsuda *et al.*, 2005). Furthermore, in zebrafish, PACAP greatly decreases the frequency of gut motility waves (Holmberg *et al.*, 2004) which might also have an impact on nutrient absorption.

npy was also upregulated in DON fed treatments, however, its putative role in our experimental setup seems to be a consequence of the reduced feed intake (Narnaware and Peter, 2001) (see review Chamorro *et al*., (2002)).

Suppression of appetite due to DON contamination in feeds might be partially explained by higher expression levels of PACAP in rainbow trout. Furthermore, the suppression of appetite might be seen as a defence mechanism to decrease the exposure of the animal to the ingested DON, therefore reducing the potential negative impacts of DON and like this explaining the lower feed intake and lack of major clinical signs.

5.3.2.3 DON metabolism

Metabolism of trichothecenes is well described in several livestock species, by the formation of de-epoxy-DON or on glucuronidation (Dänicke *et al.*, 2004; Eriksen *et al.*, 2002; Schwartz-Zimmermann *et al.*, 2015). While de-epoxy-DON is achieved mainly by gut microbiota, glucuronidation is carried out by endogenous UDP-glucuronosyltransferases in the liver, and possibly also in intestinal microsomes (Maul *et al.*, 2015). Metabolism pathways of DON vary greatly within species (Schwartz-Zimmermann *et al.*, 2015). In fish, only one report in brown bullhead catfish (*Ameriurus nebulosus*) has shown the capability of the gut microbiota of this species to biotransform trichothecenes into their de-epoxy forms (Guan *et al.*, 2009). In **Chapter 3**, is for the first time, reported that DON is metabolized into DON-3-sulfate in rainbow trout, which is less toxic than DON (Schwartz-Zimmermann *et al.*, 2015) and may explain the lack of any lesions in trout GIT when compared, for example, with swine and poultry. The location of formation, absorption and elimination of DON-3-sulfate is not known and was not further investigated in the present thesis. However, as suggested by (Schwartz-Zimmermann *et al.*, 2015), DON might be metabolized to DON-3-sulfate in the intestinal mucosa, liver, or even in the kidney as happens for some other vertebrate species. This biotransformation (DON -> DON-3-sulfate), if achieved by the gut microbiota, can also help to explain the high individual variability obtained, as the capacity to metabolize DON will be directly influenced by the individual fish microbiome. The high inter-individual variation within the groups that were fed mycotoxins highlights the importance of the individual health and nutritional status prior to DON ingestion, as supported by other authors (Hendricks, 1994). The possible individual metabolism of DON in trout helps to explain many of the results obtained during this thesis. For example, the clinical manifestation (e.g. protruding anal papilla) found in a small number of individuals as well as the lack of histological alterations in the intestines, despite the altered values of ALT and AST, in the study described in **Chapter 2**, highlights for the possible association between individual fish microbiome and its capacity to biotransform DON into DON-3-sulfate.

5.3.2.4 DON tissue distribution

Chapter 4 investigates the biological fate of DON, as it sheds light on the carryover of DON into edible tissues and provides an understanding of how to address and mitigate the impact of DON in the animal, for example by developing DON-detoxifying feed additives. Knowledge about retention time of DON in digesta, absorption, and distribution, as well as biodegradation of DON, possibly, by the indigenous GIT bacteria (described in chapter 3) are fundamental to the development of a DON-detoxifying strategy. Absorption of DON was relatively fast in rainbow trout juveniles, being the [^{3}H]-DON detected in all sampled tissues (i.e., GIT, liver, muscle, kidney and skin) just one hour after tube-feeding. The data obtained is supported by Bernhoft *et al*. (2017), which found DON in *Salmo salar* liver samples reached a maximum concentration one hour after intake. Furthermore, Bernhoft *et al*. (2017) indicate the possibility of some absorption of DON from the stomach. Rapid absorption of DON was also observed by Dänicke *et al*. (2004) and Eriksen *et al*. (2003) in pigs. This rapid absorption of DON, before reaching GIT, may be fundamental to explain the elevated PACAP gene expression (**Chapter 3**). Theoretically, rapid identification of ingested DON would be fundamental as a defence strategy of trout, in order to reduce the intake of such contaminated feed source.

In the first hour after tube-feeding, [^{3}H]-DON was also detected in the water. This indicates a relative quick passage time of [^{3}H]-DON through the GIT (< 1 hour, which is lower than the trout average gastric emptying time > 6 h; depending on temperature and meal type and size; see Langton (1977)). This may be explained by DON high water solubility, increasing its excretion with the fluid phase of the chyme. The rapid excretion of DON might also help to explain the absence of negative effects of dietary DON on the GIT. However, the high solubility and stability of DON in water may lead to re-ingestion by the fish, which needs to be further explored.

Besides the initial fast absorption of DON (at 1h, probably through stomach mucosa), the absorption during the remaining 6 hours is relatively stable which may be explained by trout's physiological condition prior to the study. Trout were fed a non-contaminated diet (during three weeks) prior to the [^{3}H]-DON tube feeding and maintained in excellent rearing conditions, presenting an optimal health condition before the trial. This

highlights the importance of the physiological conditions of the animals prior to the exposure of mycotoxins and the influence of life history on the possible consequences of such exposure, as discussed previously. It has been reported that chronic exposure to DON might cause the destruction of tight junctions (Mayer *et al.*, 2017; Pinton *et al.*, 2012) leading to increased DON absorption. For future studies, it would be interesting to understand the possible harmful effect of chronic DON exposure on the intestinal barrier and how this would influence the toxicokinetics of DON compared to the present experimental setup. The period between six and twelve hours seems to be the turning point in terms of DON excretion. Taking into account a digestion time of six hours at 15 ^{o}C (Langton, 1977) it can be expected that most of the DON were retained in the GIT and excreted after digestion. The long DON retention time in GIT, increases the probability of DON absorption (especially in case of tight junction destruction) and of a negative effect of DON on the GIT. In chapter 3, we described that more than 80% of the mycotoxin recovered from faeces was in the form of DON-3-sulfate. In experiment described in chapter 4, only DON was found as an excretion product and no DON-3-sulfate was detected. The lack of DON metabolism in the trial described in chapter 4 reinforces the possibility that the conversion of DON to DON-3-sulfate is catalysed by the gut microbiota, therefore, highly dependent on gut microbial community composition.

5.3.3 Future perspectives

The present thesis results contribute to better understand the impact of DON in rainbow trout, explaining the possible causes for the lack and/ or high variability of clinical signs, reduce feed intake and lower growth performance. It also suggests the use of DON-3-sulfate as a biomarker for DON ingestion and highlights the DON excretion time. All findings will allow bettering managing the possible risk of DON contamination in aquaculture feeds.

However, this thesis also leads us to new research questions, which would help to enhance the existing knowledge about the impact of DON in aquaculture species. In the thesis, the impact of DON on the GIT, i.e., on the absorptive process was not evaluated. The impact of mycotoxins in livestock species GIT is well described (see reviews; (Broom, 2015; Grenier and Applegate, 2013), mainly the impact of DON in tight junctions. Taking into account the mode of action of DON (as an inhibitor of protein

synthesis) and the high rate of protein turnover in intestinal cells, it was to some extent also expected to observe altered intestinal areas in trout. However, no effect of DON was observed in GIT (evaluated macroscopically (**Chapter 2** and **3**) and by histology (**Chapter 2**)) and the literature available is also not consistent when reporting the impact of DON on the trout GIT (Hooft and Bureau, 2017; Hooft *et al.*, 2011; Matejova *et al.*, 2014; Ryerse *et al.*, 2015). However, the influence of DON on nutrient absorption cannot be totally discarded and should be investigated in future.

The novel fact that 80% of the recovered DON is metabolised into DON-3-sulfate (**Chapter 3**) might help to explain the lack of GIT damage in trout, contrary to livestock species. It is crucial to further clarify the DON metabolism pathways in fish, which might be a major step toward understanding the underpinning mechanisms of sensitivity/resistance to this mycotoxin in fish and a possible solution (e.g., probiotics) for species unable to cope with DON. Would be fundamental to understand if DON is metabolised into DON-3-sulfate in other important aquaculture species.

In **Chapter 4**, was described as the excretion of DON with the fluid phase of the chyme. While the rapid excretion of DON may prevent immediate negative effects of dietary DON on the GIT, the high solubility and stability of DON in water may lead to re-ingestion by the fish. With the increasing use of recirculating aquaculture systems would be fundamental to understand the possible accumulation of DON (excreted and leached from feeds) in the system and its consequences for the animal but for the system itself, i.e., biofilter.

Conclusions

The use of plant proteins for aquaculture feeds it is a common practice across the aquaculture sector. However, evaluating mycotoxin contamination is not yet a standard practice, at least for *Fusarium* spp. mycotoxins (i.e., DON, FUM, and ZEN), being most of the commodities only tested for AF's presence. Several incorrect concepts (e.g., AF's as main mycotoxins present in aquafeeds) deeply entrenched in the aquaculture industry, might lead aquaculture companies to implement wrong mycotoxin management strategies. This ensuring a false sense of security, which may lead to economic losses due to diseases and feed inefficiency, being these losses hardly explained and rarely attributed to mycotoxins due to the difficulty to diagnose

mycotoxicoses in aquaculture species and lack of routinely Fusarium mycotoxin checks. Moreover, is important to educate the aquaculture industry that the fungi present in feeds may ultimately reduce the palatability and therefore intake of the feed, however, its presence cannot correlate with the presence of the mycotoxin and vice-versa, i.e., apparently clean feeds might contain mycotoxins (especially Fusarium mycotoxins). Furthermore, *Fusarium* spp. mycotoxins are pointed as the main mycotoxins found in shrimp and fish feeds, especially DON. As the production of these mycotoxins by *Fusarium* spp. are highly influenced by environmental conditions, is expected an increase in occurrence due to climate change. Therefore, it is imperative to understand the occurrence levels of mycotoxins in plant meals commonly used in aquafeeds.

Despite the growing existing literature on the effects of mycotoxins in aquaculture species, is still difficult to draw firm conclusions about sensitive levels for aquatic species. Published literature testing unrealistically high levels of mycotoxins, when compared to the mycotoxin contamination level found in aquaculture feeds, might convey a dangerous message to the aquaculture industry. Firstly, tends to pass the wrong idea that mycotoxin occurrence in aquafeeds is relatively low and therefore safe. Secondly, readers tend to expect the same clinical signs obtained in these studies (with high levels of mycotoxin), which might not be the same for lower contamination levels and again lead to an unrealistic sensation of safety. Actually, in **Chapter 2**, is shown that, at least in rainbow trout, the clinical manifestation of DON ingestion are dosage and exposure period dependent. Highlighting that clinical manifestation at higher contamination levels might not be extrapolated to lower contamination levels, even in the same species.

At this thesis is also concluded that short exposure periods of high levels of DON (50 days; 1,166 µg kg $^{-1}$ DON and 2,745 µg kg $^{-1}$ DON) results in a reduction in feed intake and general growth performance indicators (SGR, FCR, TGC and PER). Moreover, some clear clinical manifestations might be expected (e.g., abnormal body conformation or protruding anal papilla), however, a high inter-individuality variability response is observed. Long exposure periods to low levels of DON (168 days; 367 µg kg $^{-1}$ DON) are absolutely asymptomatic, as the animals presented no clear/specific clinical signs, being growth rate only affected after 92 days of ingesting DON. The high variability of clinical signs was reinforced by experimental data reported on **Chapter 3**, as rainbow trout fed high levels of DON during a short exposure period (11,412 ± 1,141 µg kg $^{-1}$;

60 days) did not show any macroscopic lesions (i.e., internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins).

In **Chapter 3**, was observed that trout's fed high levels of DON (11,412 ± 1,141 μg kg $^{-1}$; 60 days) saw its proteolytic enzyme activity (pepsin, trypsin and chymotrypsin) significantly altered. Trypsin may be one of the key factors explaining the lower protein turnover efficiency. Both trypsin activity and mRNA expression levels for *igf1* and *igf2*, were found to be significantly higher in the CTRL when compared to DON fed groups. DON influence on trypsin activity, and the consequent possible trypsin influence in the levels of insulin, may ultimately influence amino acid uptake, which will finally explain the lower protein retention and therefore lower growth in animals fed DON.

Decrease in trout's growth performance when fed DON, is further explained by the upregulation of *adcyap1a* or PACAP mRNAs expression levels, observed in **Chapter 3**. PACAP plays an important and direct role in the regulation of feed intake in fish, i.e., suppressing feed intake and decreasing the frequency of gut motility waves (which might also have an impact on nutrient absorption). Suppression of appetite due to DON contamination in feeds might be partially explained by higher expression levels of PACAP in rainbow trout. Furthermore, the suppression of appetite might be seen as a defence mechanism to decrease the exposure of the animal to the ingested DON, therefore reducing the potential negative impacts of DON and like this explaining the lower feed intake and lack of major clinical signs (except anorexia).

In **Chapter 3**, is also reported the DON metabolism into DON-3-sulfate, which is less toxic than DON and may explain the lack of any lesions in trout GIT. This biotransformation (DON -> DON-3-sulfate), if achieved by the gut microbiota, can also help to explain the high individual variability obtained, as the capacity to metabolize DON will be directly influenced by the individual fish microbiome. The possible individual metabolism of DON in trout helps to explain many of the results obtained during this thesis. For example, the clinical manifestation (e.g. protruding anal papilla) found in a small number of individuals as well as the lack of histological alterations in the intestines, despite the altered values of ALT and AST, in the study described in **Chapter 2**, highlights for the possible association between individual fish microbiome and its capacity to bio-transform DON into DON-3-sulfate.

Chapter 4 investigated the biological fate of DON, concluding that absorption of DON is relatively fast in rainbow trout juveniles, being the [^{3}H]-DON detected in all sampled tissues (i.e., GIT, liver, muscle, kidney and skin) just one hour after tube-feeding. This rapid absorption of DON, before reaching GIT, may be fundamental to explain the elevated PACAP gene expression (**Chapter 3**). Theoretically, rapid identification of ingested DON would be fundamental as a defence strategy of trout, in order to reduce the intake of such contaminated feed source.

The present thesis results contribute to better understand the impact of DON in rainbow trout, explaining the possible causes for the lack and/ or high variability of clinical signs, reduce feed intake and lower growth performance. It also suggests the use of DON-3-sulfate as a biomarker for DON ingestion and highlights the DON excretion time. All findings will allow bettering managing the possible risk of DON contamination in aquaculture feeds.

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Supplementary material

Table S1.1: Plant ingredients used within feeds for higher trophic level fish/shrimp species

		Plant proteins (%)						
Country	**Year**	**SBM**	**WH**	**CGM**	**R/CM**	**LKM**	**FBM**	**Other plant protein sources**
Salmons – Atlantic salmon, coho salmon, chinook salmon								
Norway	**2008**	8 to 12	10 to 14					
Norway	**2010**	12	12					Others=20
UK	**2008**	10	10		5		5	FPM=3
UK	**2010**	-	12					
Trout's – rainbow trout, sea trout								
Denmark	**2008**	12	12					
France	**2009**	10 to 15	5 to 10	5 to 8				PPM 5-10; SPC 5-10%; FPM=5-10
Greece	**2009**	10 to 35	5 to 15	5 to 12	5 to 10			FPM=5-10
Norway	**2008**	8 to 12	10 to 14					
Norway	**2010**	12	12					Others=20
UK	**2008**	15	10		5		8	FPM=3
Marine shrimps – whiteleg shrimp, giant tiger prawn								
China	**2008**	10 to 25				0 to 20		WH by-products 15-25
India	**2006/07**	20 to 25					1 to 2	G/PM=15–20
Marine fishes – barramundi, cobia, cods, groupers, halibuts, seabass, seabreams, tunas, yellowtail								
China	**2008**	10 to 25			0 to 20			WHbp=15–25
France	**2009**	15 to 25	5 to 10	10 to 18		5 to 10		PPM= 5–10, SPC=5–10
Greece	**2009**	10 to 35	5 to 15	5 to 12	5 to 10			PPM=5–10
Norway	**2010**							
Spain	**2009**		1 to 5	4	7	10		SBC=5–19; PPM=5–10
Taiwan	**2007**	15 to 25	10 to 15					
UK	**2008**	15	10					Others= 10

Abbreviations for plant ingredients stand for: Soybean Meal (SBM), Wheat (WH), Corn Gluten Meal (CGM), Rapeseed/Canola Meal (R/CM), Lupin Kernel Meal (LKM), Faba bean meal (FBM) and on other plant protein sources we have: Field Pea Meal (FPM), Pea Protein Meal (PPM), Soy Protein Concentrate (SPC), Corn (C), Soy Lecithin (SL), Cassava (CA), Rice Polishing (RP), Groundnut/peanut Meal (G/PM), Rice Bran (RB), Broken Rice (BR) and by-products (bp). Source: Adapted from Gonçalves *et al.*, 2017

Table S1.2: Plant ingredients used within feeds for lower trophic level fish/shrimp species

		Plant proteins (%)								
	Year	**SBM**	**WH**	**WB**	**C**	**CGM**	**R/CM**	**CSM**	**RB**	**Other plant protein sources**
Carps – grass carp, common carp, crucian carp, catla, rohu										
China	**2008**	0 to 25	0 to 25		0 to 25		20 to 40			SbDG=0–8%
India	**2006/07**									G/PM=30, MC=10
Catfishes – channel catfish, pangasiid catfishes										
India	**2006/07**	10	15 to 20							G/PM=30%, MC=10%
Vietnam	**2008**	30 to 60								CA=20–35
Tilapias										
Taiwan	**2007**	30 to 35							10 to 25	
Vietnam	**2008**	30 to 60							20 to 30	CA=20–35
Eels										
Denmark	**2008**	10	15							
Taiwan	**2007**	8 to 10	Starch 15–20							
Feshwater prawns										
China	**2008**			5 to 10						
India	**2006/07**	20 to 25	20 to 25							G/PM=15–20, MC=15–20
Taiwan	**2007**	15 to 20	10 to 15							

Abbreviations for plant ingredients stand for: Soybean Meal (SBM), Wheat (WH), Wheat Bran (WB), Corn (C), Corn Gluten Meal (CGM), Rapeseed/Canola Meal (R/CM), Cottonseed Meal (CSM), Rice Bran (RB) and on other plant protein sources we have: Spirit-based distillers grains (SbDG), Groundnut/Peanut Meal (G/PM), Cassava (CA), Mustard Cake (MC). Source: Adapted from Gonçalves *et al*., 2017

Table S1.3: Preferred temperatures and water activity values for fungal growth and mycotoxin production

Fungus species	Temperature range for fungal growth [°C]			Water activity (aw) for fungal growth		
	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
Aspergillus flavus	10-12	25-35	42-43	0.80	0.95-0.99	-
A. parasiticus	10-12	32-35	42-43	0.83-0.84	0.95- 0.99	-
A. ochraceus	8	24-37	37	0.77-0.79	0.95-0.99	-
Penicillium verrucosum	0	20	31-35	0.80	0.95	-
Fusarium verticillioides	2-5	22.5-30	32-37	0.87-0.9	-	0.99
F. proliferatum	4	30	37	0.9	-	-
F. culmorum	0–10	20–25	31-35	0.90-0.91	0.98-0.99	-
F. poae	5–10	20–25	35	0.90-0.91	0.98-0.99	-
F. avenacum	5–10	20–25	35	0.90-0.91	0.98-0.99	-
F. tricinctum	5–10	20–25	35	0.90-0.91	0.98-0.99	-
F. graminearum	-	24-26	-	0.9	-	0.99
F. sporotrichioides	-2	21-27.5	35	0.88	-	0.99
Claviceps purpurea	9-10	18-22	-	-	-	-

Fungus species	Water activity (aw) for mycotoxin formation			Temperature range for mycotoxin formation [°C]		
	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
Aspergillus flavus	0.82	0.99-0.99	0.99	12-15	30-33	37-40
A. parasiticus	0.87	0.99	-	12	33	40
A. ochraceus	0.80-0.85	0.98	-	12-15	25-31	37
Penicillium verrucosum	0.83-0.86	0.90-0.95	-	4	20-25	-
Fusarium verticillioides	0.92-0.93	-	-	10	15-30	37
F. proliferatum	0.93	-	-	10	15-30	37
F. graminearum	0.9-0.91	0.98	-	11	29-30	-
F. culmorum	-	-	-	11	29-30	-

Table S1.4: Revision of literature on the effects of Aflatoxins in fish species.

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Nile tilapia (*Oreochromis niloticus*)	(0, 0.25[1] µg kg $^{-1}$) x Vit or C	Hessein *et al.* 2014	n/a	n/a	Y[1] ↓WG ↓FCR	Y[1] ↓TP ↓alb ↑ALT ↑AST ↑Urea-N	N	M[1]=101.7 µg kg $^{-1}$	● Initial weight: 7.3 g; 98 days study ● Tested coumarin (5 g kg $^{-1}$ diet; C) and vitamin E (50mg Kg $^{-1}$ diet; Vit) as detoxifying strategy ● No differences on Hb, RBcs, Hct, WBCs, Plat
African sharptooth catfish *(Clarias Gariepinus)*	10[1], 17[2] and 20[3] µg AFB_1 Kg $^{-1}$	Suzy *et al.* 2017	n/a	n/a	↓FC[2] ↓LW[2,3] ↓WG[2,3] ↓TL[2,3] ↓SL[2,3] ↓SGR[2,3]	N	n/a	M[1]= 0.05±0.12 µg AFB_1 Kg $^{-1}$ M[2]= 0.08±0.10 µg AFB_1 Kg $^{-1}$ M[3]= 0.08±0.12 µg AFB_1 Kg $^{-1}$	● Initial weight: 4±2 g; 3 months study ● Chicken droppings was used as ingredient contaminated with 5; 7.2 and 8.2 µg AFB_1 Kg $^{-1}$ ● Catfish fed 10 AFB_1 Kg $^{-1}$ used as control ● No differences in haematological parameters
Beluga *(Huso huso)*	0, 25, 50, 75[1] and 100[2] µg AFB_1 Kg $^{-1}$	Sepahdari *et al.* 2010	L[1,2]	n/a	↑FCR[1,2] ↓WG[1,2]	n/a	N	n/a	● Initial weight: 120±10g; 3 months study ● Liver: fat deposition, hepatocyte degeneration and necrosis[1,2], after 60 days
Beluga *(Huso huso)*	10 µg AFB_1 Kg $^{-1}$	Farabi *et al.* 2006	Haemr SK Haemr Ab Haemr Hd GB L	n/a	↓AGR	n/a	↑ (8.6%)	n/a	● Initial weight: 1,850 ± 129.3 g, 15 days study ● Unintentionally exposed to contaminated feed by AF during 15 days. ● Clinical signs: Haemorrhagic skin lesions in the head and abdominal regions; distortion of the spinal column and yellow spots in pectoral fins region; Hyperinflation of gallbladder

Channel catfish *(Ictalurus punctatus)*	Control 16[1] µM AFB_1 128[2] µM AFB_1	Gallagher and Eaton, 1995	L[1,2]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 50 - 75 g ● In vitro AFB_1 biotransformation in hepatic microsomes ● AFB_1 is poorly oxidized by channel catfish liver microsomes
Channel catfish *(Ictalurus punctatus)*	0; 100; 464; 2,154 and 10,000[1] µg AFB_1 Kg^{-1}	Jantrarotai and Lovell 1990	L[1], HK[1] HT[1], I[1] SG[1]	n/a	↓GR[1]	Y[1] ↓Hct, ↓Hb, ↓Ery, ↑HTa	n/a	n/a	● Initial weight: 7.5 g; 10 weeks study ● [1]Necrotic stomach gastric glands
Channel catfish *(Ictalurus punctatus)*	Control; [#1]550 µg AFB_1 Kg^{-1} [#2]275 µg AFB_1 Kg^{-1} [#2]134 µg AFB_1 Kg^{-1}	Manning *et al.* 2005	N	N	N	N	N	n/a	● Initial weight: 7.1 g[#1]; 50 g[#2] ● AF natural contamination
Coho salmon *(Oncorhynchus kisutch)*	100 and 500 µg kg^{-1} [^{3}H] AFB_1	Nakatsuru *et al.* 1989	↑AFB_1-DNA adduct level[1]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 150-170 g ● Intravenously injection of AFB_1 in first day of experiment; fishes killed 9 hours after
Common carp *(Cyprinus carpio)*	0, 50[1], 100[2] µg $kg^{-1}$$AFB_1$	Akter *et al.* 2011	n/a	n/a	↓WG[2; Ti-Tf] =SGR[1,2] =FCR[1,2]	n/a	N	M[1]= [Ti]1.56±0.18; [Tf]1.28±0.12 µg kg^{-1} AFB_1 M[2]= [Ti]2.08±0.08; [Tf]1.65±0.11 µg kg^{-1} AFB_1 L[1]= [Ti]3.10±0.10; [Tf]2.92±0.07 µg kg^{-1} AFB_1 L[2]= [Ti]2.14±0.11; [Tf]2.27±0.09 µg $kg^{-1}$$AFB_1$	● Initial weight: 13.46 ± 2.98 g ● 30[Tf] days study with sampling at 15 [Ti] days
Common carp *(Cyprinus carpio)*	0; 500[1]; 700[2]; 1,400[3] µg kg^{-1} AFB_1	Banaee *et al.* 2017	L[1-3] K[1-3] G[1-3] I[1-3]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 30±5 g ● 21 days study
European seabass (*Dicentrarchus labrax* L.)	[#1]Oral 96 h LC_{50} >50, 100, 150, 200, 250, 300, 350 and 400 µg kg^{-1} bwt	El-Sayed and Khalil 2009	L[1,2] K[1,2] SK[1,2] G[1,2] GB[1,2]	n/a	n/a	[#2]Y ↑ALT ↑AST ↑ALP ↓TP,	[#2]N	[#2]M= 5 µg AFB_1 Kg^{-1}	● Initial weight: 40±2 g ● [#1]96h LC_{50} = 0.18 mg kg^{-1} bwt ● [#2]Intake: 18 mg kg^{-1} bwt AFB_1 (10% of oral 96h) ● [#1,2] Clinical signs: sluggish movement,

	[#2]42 days exposure to 10% of oral 96h LC_{50}= 180 µg kg $^{-1}$ bwt					↓alb, ↓glob			loss of equilibrium, rapid opercular movement, and hemorrhages of the dorsal skin surface. [#2]Yellowish discoloration, pale discoloration of the gills, liver and kidney. Severe distension of the gall bladder.
Gibel carp *(Carassius gibelio)*	3.2, 11.3, 20.2[1], 55.2[2], 95.8[3], 176.0[4],991.5[5] µg AFB_1 kg $^{-1}$	Huang *et al.* 2011	N	n/a	N	N (ALT, AST, ALP, SOD, TP)	N	HP[1-5] >5 µg AFB_1 Kg $^{-1}$ M[5]=2.35 µg AFB_1 Kg $^{-1}$	● Initial weight: 10.33±0.19 g, ● 12 weeks study ● Fish showed strong clearance ability of AFB_1
Gibel carp *(Carassius gibelio)*	3.3[1], 22.3[2], and 1,646.5[3] µg AFB_1 kg $^{-1}$	Huang *et al.* 2014	↓GSI[2,3] ↓AF[2,3] ↓RF[2,3] ↓Ood[2,3]	n/a	N (Fr, SGR, FBW, FE)	N (ALT, AST)	N	O[1]= 1.20 µg AFB_1 Kg $^{-1}$ O[2]= 4.12 µg AFB_1 Kg $^{-1}$ O[3]= 5.32 µg AFB_1 Kg $^{-1}$ M[2] = 3.11 µg AFB_1 Kg $^{-1}$ M[3] = 4.0 µg AFB_1 Kg $^{-1}$	● Initial weight: 57.6 ± 0.1 g ● 24 weeks study
Gibel carp *(Carassius gibelio)*	3.20[1], 5.37[2], 7.08[3], 9.55[4], 12.70[5], 17.90[6] and 28.60[7] µg AFB_1 kg $^{-1}$	Han *et al.* 2010	N	n/a	↓WG [t1;6,7] ↓WG [t2;6] ↓WG [t3;6] =HSI; =FCR ↑CF [t3;3,4;6,7]	=CAT [Se] =AST [HP] ↑SOD[Se;4-7] ↑ALT [HP;7]	N	O[4-6]>2 µg AFB_1 Kg $^{-1}$ M[4-6] > 2 µg AFB_1 Kg $^{-1}$ O[7]=3.16 µg AFB_1 Kg $^{-1}$ M[7] = 4.08 µg AFB_1 Kg $^{-1}$	● Initial weight: 3.53 ± 0.02 g ● 12 weeks study (4 weeks [t1], 8 weeks [t2], 12 weeks [t3])
Gilthead sea bream (*Sparus aurata*)	From 5 x 10 ^{3} ng ml $^{-1}$ to 2 x 10 $^{-5}$ ng ml $^{-1}$ of AFB_1	Centoducati *et al.* 2010	L	n/a	n/a	n/a	n/a	n/a	● Primary monolayer cultures of hepatocytes ● Exposure = 4, 48, 72 hours. ● Exhibited dose- and time-dependent cytotoxic effect, the IC_{50} being inversely related to the exposure time (MTT-IC_{50}-24h, 5x10^3 ng/ml; MTT-IC_{50}-48h, 6x10^2 ng ml $^{-1}$; MTT-IC_{50}-72h, 60 ng ml $^{-1}$)
Hybrid sturgeon (*Acipenser ruthenusx A. baeri)*	0, 1, 5, 10, 20[1], 40[2] and 80[3] µg AFB_1 kg $^{-1}$	Rajeev Raghavan *et al.* 2011	L[2,3]	n/a	N	n/a	Y[1-3]	M≈28[2] and 34[3] L= 142.80[2] and 115.60[3] ng g $^{-1}$	● Initial weight: 10.53 ± 0.17 g ● 35 days study ● Liver hypertrophy and hyperchromasia of nuclei and cytoplasmic vacuoles, presence of inflammatory cells, focal hepatocyte necrosis and extensive biliary

									hyperplasia.
Hybrid tilapia (Oreochromis niloticus ×O. aureus)	19; 85[0]; 245[1]; 638[2]; 793[3] and 1,641[4] µg kg $^{-1}$	Deng *et al.* 2010	YS[3,4>t1] L[t;2-4]	n/a	Y[t; 1-4] ↓WG ↓FI ↓FER	Y [t;2-4] ↓TP, ↓alb =ALT, =AST =ALP	N	Y [t1+t;0-4] L [t1]=10[0], 16[1], 21[2], 24[3] and 24[4] µg AFB_1 kg $^{-1}$ liver L [t]=30[0], 33[1], 47[2], 44[3] and 43[4] µg AFB_1 kg $^{-1}$ liver	● Initial weight: 20 g; ● 20[t] weeks study (sampling at week 5[t1]) ● AF from mouldy peanut meal
Jundiá (*Rhamdia quelen*)	[#1] 41, 90[1] and 204[2] µg AFB_1 kg $^{-1}$ [#2]350[1]; 757[2]; 1,177[3] µg AFB_1 kg $^{-1}$	Lopes *et al.* 2005	[#1]L[2] [#2]L	n/a	n/a	n/a	N	[#1]M =1[1] and 6.1[2] µg AFB_1 Kg^{-1} [#2]M+L=350[1]; 757ppb[2] and 1,177[3] µg AFB_1 Kg^{-1}	● Initial weight: 3.21[#1] g and 4.73[#2] g ● 45[#1] and 35[#2] days study
Jundiá (*Rhamdia quelen*)	(0, 150[1], 250[2] e 350[3] µg AFB_1 kg $^{-1}$) x (0,3 ou 0,6% ad)	Lopes *et al.* 2009	n/a	n/a	↓FW[1-3] ↓CF[1-3]	n/a	N	n/a	● Initial weight: 4.2 ± 0.6 g ● 90 days study ● Tested an aflatoxin adsorbent (Sodium and calcium aluminium silicate); adsorbent did not alleviate AF negative effects
Lambari fish (*Astyanax altiparanae*)	0, 10[1], 20[2] and 50[3] µg AFB_1 kg $^{-1}$	Michelin *et al.* 2016	n/a	n/a	n/a	n/a	n/a	L=265[2,t] and 243[3,t] µg kg $^{-1}$ M=19[1,t], 20[2,t] and 50[2,t] µg kg $^{-1}$	● Initial weight: 3.15 g ● 120 days study (sampling at day 30, 60, 90 and 120[t]) ● For the first 60 days of exposure, AFs were metabolised by liver and excreted, after 90 days, a lower efficiency in the elimination of AFs
Mozambique tilapia *(Oreochromis mossambicus)*	0; 375[1]; 2,500[2]; 6,000[3] µg AFB_1 kg $^{-1}$	Varior & Philip 2012	L[2]	n/a	n/a	↓nAPh[1-3] ↓lAPh[1-3] ↓sAPh[1,3]	n/a	n/a	● Initial weight: 10±3g; 6 weeks study ● AFB_1 grown on polished raw rice as substrate ● Liver[2] showed complete necrosis of the hepatic cells
Nile tilapia (*Oreochromis niloticus*)	(0 and 200[1] µg AFB_1 kg $^{-1}$) x (FEO + SC)	Abdel Rahman *et al.* 2017	n/a	n/a	n/a	↓Ph-aw[1] ↓PhI[1] ↓TP[1] ↓Alb[1] ↓Glob[1] ↑ALT[1] ↑Creat[1]	n/a	L[1]= 5±0.5 µg AFB_1 kg $^{-1}$ M[1]= 3.7±0.1 µg AFB_1 kg $^{-1}$	● Initial weight: 26.6±0.12 g; 30 days study ● Tested fennel essential oil (FEO) and saccharomyces cerevisiae (Sc) as mycotoxin management strategy. ● AF effects are reported only for 0 and 200[1] µg kg $^{-1}$

Nile tilapia (*Oreochromis niloticus*)	0; 940; 1,880[#1]; 375; 752[1]; 1,500[2], 3,000[3] µg kg $^{-1}$	Chávez-Sánchez *et al.* 1994;	L[2,3] K[2,3] G[1-3]	n/a	Y[1-3] ↓FI ↓WG ↓SGR	n/a	N	n/a	● Initial weight: 0.5 g ● 25 days study with extra 50 days recover period; sampling at day 15, 26, 54 and 75 ● Fatty liver and characteristic neoplastic changes such as nuclear and cellular hypertrophy, nuclear altrophy, increase in number of nucleoli, cellular infiltration, hyperemia, cellular basophilia and necrosis. [#1]AF value seems an error in the manuscript
Nile tilapia (*Oreochromis niloticus*)	0; 1.5[0]; 0+B[1]; 0+M[2]; 1.5+B[3] and 1.5+B[4] µg kg^{-1}	Hassan *et al.* 2010	L[0], T[0]	n/a	n/a	Y[0] ↓TP, ↓alb ↓glo ↓test	n/a	n/a	● Initial weight: 90±10 g; 3 weeks study ● Tested a bentonite (B) and a montmorillonite (M) clay (5g kg $^{-1}$ diet) ● AF effects are reported only for 1.5[0] µg kg $^{-1}$
Nile tilapia (*Oreochromis niloticus*)	350; 757; 1,177[1] µg kg $^{-1}$	Oliveira *et al.* 2013	n/a	n/a	N[#1] Y[#2] ↓FCR[1] ↓TL[1]	n/a	N	n/a	● Initial weight: 1.55±0.005g [#1]; 30 days study ● Challenge test[#2] with *Aeromonas hydrophila* ● Authors suggest a synergism between AFB_1 and *Aeromonas hydrophila.*
Nile tilapia (*Oreochromis niloticus*)	0; 250[1]; 2,500[2]; 10,000[3] or 100,000[4] µg AFB kg $^{-1}$	Tuan *et al.* 2002	L[3,4] N[1-4] (Sp, S, I, K, H)	n/a	Y[2-4] ↓WG	Y[2-4] ↓Hct	Y[4]	n/a	● Initial weight: 2.7g; ● 8 weeks study
Nile tilapia (*Oreochromis niloticus*)	(0; 1,500[1] and 3,000[2] µg AFB_1)x 0% CM, 0.5% CM and 1.0% CM	Zychowski *et al.* 2003b	L[1,2]	Y[1,2]	Y[1,2] ↓WG ↓FI ↓HSI	Y[1,2] ↓MESa	N	n/a	● Initial weight: 2 ±0.1 g; 10 weeks study ● Tested an calcium montmorillonite (CM) clay ● 0.5 and 1% CM included in diets containing 1.5 ppm AFB_1 decreased total histopathological impact however; this protective effect was not evident when fish were exposed to 3.0 ppm AFB_1.
Nile tilapia (*Oreochromis niloticus*)	(0, 250[1] µg AFB_1 kg feed $^{-1}$) x OZ, B or C	Ayyat *et al.* 2013	n/a	n/a	Y[1] ↓WG ↓FCR	Y[1] ↓RBCs ↓Plat ↓TP, ↓alb,	N	M[1]=78.33 µg kg $^{-1}$	● Initial weight: 7.3 g; 3 weeks study ● Tested ozone (0.5 mg/L/minute; OZ), bentonite (20 g kg $^{-1}$ diet; B) and coumarin (5 g kg $^{-1}$ diet; C) as detoxifying strategy

						↑ALT, ↑AST, ↑Urea-N			
Nile tilapia (*Oreochromis niloticus*)	[#1]<5[i] - 38.62[f] µg kg [-1] [#2]28.82 [i] -72.39 [f] p µg kg [-1] [#3]53.02 [i] -115.34 [f] µg kg [-1]	Cagauan *et al.* 2014	L[#1-3] SK[#1-3] YS[#1-3] BD[#1-3] RF[#1-3]	n/a	N	N	Y[#1-3]	n/a	● Initial weight: 30-40 g; 90 days study ● Naturally AF contaminated feed used ● AF contamination in feed was not constant during trial; [i]=initial; [f]=final)
Nile tilapia (*Oreochromis niloticus*)	(0, 2[1], 4[2] µg kg [-1]) x 0.5% CB	Hussain & Mateen 2017	n/a	n/a	Y[1,2] ↓SGR ↓WG ↓FER ↓FI ↓PER	n/a	Y[1,2]	n/a	● Initial weight: 4.5±0.4g; 10 weeks study ● Tested calcium bentonite (CB) clay as detoxifying strategy; ● Tested CB significantly improved some parameters
Nile tilapia (*Oreochromis niloticus*)	(0, 2[1], 4[2] µg kg [-1]) x 0.5% and 1% CB	Hussain *et al.* 2017	n/a	n/a	Y[1,2] ↓HSI ↓WG ↓FER ↓FI ↓PRE ↓CLRE ↓MR	n/a	N	M[2]=87±1.32 ng g [-1]	● Initial weight: 4.5±0.4g; 10 weeks study ● Tested calcium bentonite (CB) clay as detoxifying strategy; ● Tested CB significantly improved some parameters (WG, HIS) ●CB significantly reduced bioaccumulation of AFB_1 residues in muscle tissues.
Nile tilapia (*Oreochromis niloticus*)	20[1] and 100[2] µg AFB1 kg [-1] feed	Mahfouz *et al.* 2015	L[2]	Y[1,2]	Y[2; t1, t2] ↓WG ↓FI ↓HSI	Y[2 t1, t2] ↓HSI ↑ALT ↑AST ↑ALP ↓WBCs ↓Hb	Y[1,2]	L [1,t1]=5 µg kg [-1] [1,t2]=8 µg kg [-1] [2,t1]=10 µg kg [-1] [2,t2]=15 µg kg [-1] M[2; t2]=5 µg kg [-1]	● Initial weight: 35±0.50g; 6[t1] or 12[t2] weeks study ● Challenge test with *Aeromonas hydrophila, IP* ● Expression of liver *GPx* and *GST* down-regulated[1] ● The ability to withstand *A. hydrophila* infection was remarkably lowered
Nile tilapia (*Oreochromis niloticus*)	(0 and 100[1] µg kg [-1]) x BB, CF, A and G	Mehrim *et al.* 2006	L[1] K[1] I[1] G[1]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 10g; 14 weeks study ● Tested BioBuds-2x (BB), chamomile flowers (CF), Aspirin (A) and Ginger (G) (inclusion=0.5%) as detoxifying strategy;
Nile tilapia (*Oreochromis niloticus*)	(0 and 200[1] µg kg [-1]) x HSCAS, SC and EGM	Selim *et al.* 2014	n/a	n/a	Y[1] ↓WG ↓ADG	Y[1] ↓RBCs ↓Hb	Y[1]	M[1]≈90 µg kg [-1]	● Initial weight: 15±2g; 10 weeks study ● Tested hydrated sodium calcium aluminosilicates (HSCAS; 0.5%),

					↓SGR	↑WBCs ↑AST ↑ALT ↓TP ↓alb ↓glob			*Saccharomyces cerevisiae* (S.C.; 0.25%) and an esterified glucomannan (EGM; 0.25%) as detoxifying strategy; ● AF produced from polished raw rice
Nile tilapia (*Oreochromis niloticus*)	0, 150[1] µg AFB_1 kg^{-1}	Salem *et al.* 2009	n/a	n/a	↓WG[1] ↓ADG[1] ↓SGR[1] ↑FI[1] ↑FCR[1] ↓PER[1] ↓PPV[1]	↑WBCs[1] ↑AST[1] ↓ALT[1]	Y[1]	M= 99.48 µg AFB_1 kg^{-1}	● Initial weight: 10±3g; 15 weeks study ● AFB_1 was produced through pellets fermentation using *Aspergillus parasiticus* NRRL 2999
Nile tilapia (*Oreochromis niloticus*)	15.6 µg ml^{-1} of AFB_1	Ngethe *et al.* 1993	n/a	n/a	n/a	n/a	n/a	L[1, 2, 4] B[1, 2, 4]	● Initial weight: 200±20g; 3 weeks study (sampling at 6h[1], 1 day[2], 2 days[3] and 6 days[4]) ● Intravenously injection of ^3H-AFB_1
Nile tilapia (*Oreochromis niloticus*)	0; 20[1]; 200[2]; 2,000[3] µg AFB_1 kg^{-1}	Marijani *et al.* 2017	[M,F]L[1-3]	n/a	n/a	↑[M,F]HSI[1-3] ↑[M,F]KSI[1-3] ↑[M,F]SSI[2, 3] ↑[M,F]WBC[2,3] ↓[M,F]Mono[1-3] ↑[M,F]Neut[1-3] ↑[M,F]Lyph[1-3] ↑[M,F]ALT[2,3] ↓[M,F]RBCs[1-3] ↓[M,F]Hb[1-3]	n/a	n/a	● Initial weight: 24.1±0.6 g ● Results from sampled parameters were differentiated from males[M] and females[F].
Rainbow trout	0 and 80,000[1] µg kg^{-1} of AFB_1	Arana *et al.* 2013	L[1]	n/a	n/a	n/a	N	n/a	● Initial weight: 18g; 12 months study ● 6 trout's sampled monthly

(*Oncorhynchus mykiss*)									
Rainbow trout (*Oncorhynchus mykiss*)	[#1]0.01[1], 0.025[2], 0.05[3], 0.1[4], 0.25[5], 0.5[6] µg kg $^{-1}$ AFB_1 [#2]4[1], 8[2], 16[3], 32[4], 64[5] µg kg $^{-1}$ AFB_1	Bailey *et al.* 1994	[#1]↑Hti[1-6] [#2]↑Hti[1-5]	n/a	n/a	n/a	[#1]Y[3,6] [#2]Y[1,5]	n/a	● [#1]21 day old embryos exposed by bath to AF for 1 h ● [#2] Dietary supplementation of AFB_1 for 9 months
Rainbow trout (*Oncorhynchus mykiss*)	[#1]0.01[1], 0.025[2], 0.05[3], 0.1[4], 0.25[5], 0.5[6] µg kg $^{-1}$ AFL [#2]4[1], 8[2], 16[3], 32[4], 64[5] µg kg $^{-1}$ AFB_1	Bailey *et al.* 1994	[#1]↑Hti[1-6] [#2]↑Hti[1-5]	n/a	n/a	n/a	[#1]Y[3,4] [#2]Y[1,3-5]	n/a	● [#1]21 day old embryos exposed by bath to AF for 1 h ● [#2] Dietary supplementation of AFB_1 for 9 months
Rainbow trout (*Oncorhynchus mykiss*)	20 µg kg $^{-1}$ AFB_1 and 20 µg kg $^{-1}$ AFB_1 + 2% clay	Ellis *et al.* 2000	n/a	n/a	n/a	n/a	n/a	F, K, GI, U, Bi, Ca	● Initial weight: 266±12.6 g, 7 days study ● 2% sodium bentonite Volclay tested as detoxifying strategy;
Rainbow trout (*Oncorhynchus mykiss*)	15.6 µg ml $^{-1}$ of AFB_1	Ngethe *et al.*, 1992	n/a	n/a	n/a	n/a	n/a	Bi, L, K, B, AbF, M, Sp and Bl	● Initial weight: 100±15 g, 8 days study (sampling at 6h, 1, 2 4 and 8 days) ● Intravenously injection and Oral dose of ^{3}H-AFB_1
Rainbow trout (*Oncorhynchus mykiss*)	0 to 1,000 µM AFB_1	Ottinger and Kaattari, 1998	n/a	y	n/a	↓ Lyph Pr ↓Ig P	n/a	n/a	● Initial weight: 250–500 g ● Trout leucocyte *in vitro* assay ● Assays evaluated during the 18 months (June 1985–November 1996) ● Immunosuppression was observed at aflatoxin doses lower than those resulting in toxicity
Rainbow trout (*Oncorhynchus mykiss*)	0 or 5,000[#1] µg L $^{-1}$ AFB_1	Ottinger and Kaattari, 2000	n/a	y	n/a	↓ Lyph Pr ↓Ig P ↑Leuc	n/a	n/a	● Initial weight: 50–150 g ● [#1]Embryos exposed to aflatoxin B_1 for 30 min ● Challenged with LPS at 10 mg l $^{-1}$
Rainbow trout (*Oncorhynchus mykiss*)	Single IP injection of [^{3}H] AFB_1 (30 µCi/ 10 µg kg $^{-1}$ body weight) after fed #1, #2 or #3	Takahashi *et al.* 1995	n/a	y	n/a	↑hepatic AFB_1-DNA binding ↑EROD	n/a	n/a	[#1]Experiment 1; trout's fed AFB1 diets containing Indole-3-carbinol (I3C)[I3C] or β-naphthoflavone (BNF)[BNF] during 1 week; after fish were sacrificed and livers used for microsomes assay. [#2]Experiment 2; Trout's (5-10g body weight) were fed diets for 7 days; posterior

									IP [^{3}H]AFB$_1$ injection #1(1000K, 2000K, 3000K or 4000K µg kg $^{-1}$)I3C or (100K or 500K µg kg $^{-1}$) BNF #2; 13C 0, 500K, 1000K, 2000K, 3000K or 4000K µg kg $^{-1}$ #3 Experiment 3; 126 trout (1-2 g body weight) fed 2000K I3C; IP [^{3}H]AFB$_1$ injection after 1, 2, 3, 5 or 7 days of feeding.
Rainbow trout (*Oncorhynchus mykiss*)	0 t1; #1115 t2; #2347 t2; #31,190 t2; #46,276 t3 µg kg $^{-1}$ AFB$_1$ and #5(219 AFB$_1$+306 AFB$_1$+321 AFG$_1$+270 AFG$_2$) t3	Nomura *et al.* 2011	n/a	n/a	n/a	n/a	n/a	#1M=90 c3; 90 c7; 100 c14; 60 c21 ng kg $^{-1}$ AFB$_1$ #2M=200 c3; 210 c7; 230 c14; 170 c21 ng kg $^{-1}$ AFB$_1$ #3M=490 c3; 720 c7; 880 c14; 260 c21 ng kg $^{-1}$ AFB$_1$ #4M=4100 c7 ng kg $^{-1}$ AFB$_1$ #5M=150 c7 ng kg $^{-1}$ AFB$_1$	● Initial weight: 2.1±0.1 g ● Experimental period: 23 t1; 2 t2 or 7 t3 days ● Carry over evaluated at 3 c3, 7 c7, 14 c14 and 21 c21 days ● "AFs are eliminated rapidly and are not biomagnified in fish"; Nomura *et al.* 2011 ● AF was detected in the rearing water
Rainbow trout *(Oncorhynchus mykiss)*	1 5.8 µg kg $^{-1}$AFB$_1$; 2 5.9 µg kg $^{-1}$AFM$_1$ and 3 27.3 µg kg $^{-1}$AFM$_1$	Canton *et al.* 1975	K T2-T7;1-3 L T2-T7;1-3 S T2-T7;1-3	n/a	↓WG >T5; 2,3	n/a	n/a	n/a	● Initial weight: 25 g ● 16 months study; samplings for WG at 3 T1, 5 T2, 7 T3 and 12 T5 months; sampling for K, L and S histopathology at 5, 9 T4, 12 T6 and 16 T7 months.
Rainbow trout *(Oncorhynchus mykiss)*	100 1 and 500 2 µg kg $^{-1}$ [^{3}H]AFB$_1$	Nakatsuru *et al.* 1989	↑AFB$_1$-modified DNA 1,2 ↑AFB$_1$-DNA adduct level 1,2	n/a	n/a	n/a	n/a	n/a	● Initial weight: 30-50 g ● Intravenously injection of AFB$_1$ in first day of experiment; fishes killed 24 hours after
Red Drum (*Sciaenops ocellatus*)	0; 100 1; 250 2; 500 3; 1,000 4; 2,000 5; 3,000 6 or 5,000 7 µg kg $^{-1}$ AFB$_1$ (+5,000 µg kg $^{-1}$ + 1%CMC)	Zychowski *et al.* 2013	L 3-7	n/a	Y ↓FE 1,2, 5-7	Y ↓Lyz 1-7 ↓Try 1,3-7 ↓HSI 5,7	Y 1,2, 5-7	n/a	● Initial weight: 2.1±0.1 g, 7 weeks study ● Tested a calcium montmorillonite clay (CMC) as detoxifying strategy;
Rohu (*Labeo rohita)*	50, 100 and 150 1 µg kg $^{-1}$ AFB$_1$	Baglodi *et al.* 2015	N	N	↓SGR 1	n/a	N	n/a	● Initial weight: 1.5 g; 3-4 months fingerlings

									● 130 days study ● SGR started to slowly be reduced near 130 days of study for rohu fed 150 µg kg^{-1} AFB_1
Rohu (*Labeo rohita)*	0, 20% AB, 100 µgAFB_1/100gDW[1] and 100µgAFB_1/100g DW + 20%AB	Madhusudhanan *et al*. 2004	L[1] K[1] B[1]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 175-250 g, 7 weeks study ● Intravenously injection and Oral ● Study on protective effects of *Amrita Bindu* (AB) increase in conjugated diene formation and LPO not only in liver but also in kidney and brain
Rohu (*Labeo rohita)*	Control, 10% MF[1], 50% MF[2] and 100% MF[3]	Ruby D. S *et al.* 2013	n/a	n/a	Y ↓FBW[1-3] ↓SGR[1-3]	n/a	Y[1-3]	n/a	● Initial weight: 30-50 g ● AF produced from mouldy feed ● AF contamination values were not determined. Authors projected the experiment with inclusion of mouldy feed (MF) ● The authors did not treated data statistically
Rohu (*Labeo rohita)*	0; 1,250[1]; 2,500[2] and 5,000[3] µg kg^{-1} AFB_1	Sahoo and Mukherjee, 2001a	n/a	Y*[1-3]	n/a	Y ↓NTB[2,3] ↓TP[1-3] ↓glob[1-3] ↓A:G[1-3]	Y ↑[1] ↓[2,3]	n/a	● Initial weight: 30-50 g, 90 days study ● Intravenously injection of AFB_1 and observed in the subsequent 90 days; ● Bacterial agglutination titre with *Edwardsiella tarda**
Rohu (*Labeo rohita)*	0; 1,250[1] and 5,000[2] µg kg^{-1} AFB_1 BW	Sahoo *et al.* 2001b	n/a	N*[1,2] B, L, K, G, H	n/a	Y ↓NTB[2] ↓TP[1,2] ↓Alb[2] ↓glob[2] ↑A:G[1-3]	n/a	n/a	● Initial weight: 30-50 g, 90 days study ● Intravenously injection of AFB_1 and observed in the subsequent 90 days; ● Bacterial agglutination titre with *Edwardsiella tarda**
Rohu (*Labeo rohita)*	1,250 µg kg^{-1} AFB_1	Sahoo and Mukherjee, 2001c	n/a	n/a	n/a	↑A:G ↑Bact.aw ↑Lyz ↓NBT	y	n/a	● Initial weight: 30 -55 g; 60 days study ● Intravenously injection of AFB_1 in first day of experiment ● Tested levamisole hydrochloride as immune stimulant
Rohu (*Labeo rohita)*	1,250 µg kg^{-1} AFB_1	Sahoo *et al.* 2001d	n/a	n/a	n/a	↓HbT ↓BactAT ↓HaeL	y*	n/a	● Initial weight: 39g; 60 days study ● Intravenously injection of AFB_1 in first day of experiment and observed in the subsequent 60 days

									● Tested β-1,3 glucan as immune stimulant; adde to the feed at 0.1% ● Fish were challenged by IP injection with formalin-killed *Edwardsiella tarda**
Rohu (*Labeo rohita)*	1,250 µg kg $^{-1}$ AFB_1	Sahoo *et al.* 2002	n/a	n/a	N	↓HbT ↓BactAT ↑Bact.aw ↓Lyz ↓Glob ↓NBT	N	n/a	● Initial weight: 30 - 35 g; 60 days study ● Intravenously injection of AFB_1 in first day of experiment ● Tested α-tocopherol (1000 mg kg $^{-1}$) ● Fish were challenged by IP injection with formalin-killed *Aeromonas hydrophila* and *E. tarda*.
Rohu (*Labeo rohita)*	1,250 µg kg $^{-1}$ AFB_1	Sahoo *et al.* 2003	n/a	n/a	y*	↓HbT ↓BactAT ↓HaeL ↑Bact.aw ↓Lyz	y*	n/a	● Initial weight: 30 - 35 g; 60 days study ● Intravenously injection of AFB_1 in first day of experiment ● Fish were challenged by IP injection with live *E. tarda* y* ● Diets supplemented with L-ascorbyl-2-polyphosphate at 15%
Rohu (*Labeo rohita)*	(0, 10[1], 20[2], and 40[3] µg kg $^{-1}$$AFB_1$) x with or without mold inhibitor	Mohapatra *el at*., 2011	L[3] K[2,3] G[2,3]	n/a	n/a	↓RBC[1-3] ↑WBC[1-3] ↓Hb[1-3] ↓NTB[1-3] ↓TP[1-3] ↓alb[1-3] ↓glob[1-3] ↓A:G[1-3]	n/a	n/a	● Initial weight: 1.48 -1.54 g, 60 days study ● Tested beside the AFB_1 levels, the action of a mould inhibitor composed by 0.25% clove oil + 0.32% sodium propionate.
Silver catfish *(Rhamdia quelen)*	0; 1,177 µg kg $^{-1}$$AFB_1$	Balsidera *et al.* 2018 a	↑B [T1] ↑↑B [T2] ↑↑↑B [T3] BBB [T1-T3] ↑AChE [T1-T3] ↓/P p.aw [T1-T3]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 90.32 ± 7.54 g ● Fish were fed experimental diets and evaluated at 7[T1], 15[T2] and 21[T3] days ● Behaviour impairment on AFB_1 fed animals associated with hyperlocomotion [T2,T3] ● "Hyperlocomotion may be considered an important macroscopic marker indicating possible AFB_1 intoxication" , Balsidera *et al*., 2018
Silver catfish *(Rhamdia*	0; 1,177 µg kg $^{-1}$$AFB_1$	Balsidera *et al.* 2018 b	n/a	n/a	n/a	↓Cc.CK. aw [T2,T3]	n/a	n/a	● Initial weight: 90.32 ± 7.54 g ● Fish were fed experimental diets and

quelen)						↑Mcd.CK.aw [T1-T3] ↓Cc.AK.aw [T2,T3] ↓Cc.PK.aw [T2,T3] ↓ATP [T2,T3]			evaluated at 7[T1], 15[T2] and 21[T3] days
Silver catfish *(Rhamdia quelen)*	0; 1,177 µg kg $^{-1}$AFB$_1$	Balsidera *et al.* 2018 c	n/a	n/a	n/a	↑XO.aw [T2,T3] ↑UA [T2,T3] ↑NO [T2,T3] ↑ROS [T2,T3]	n/a	n/a	● Initial weight: 90.32 ± 7.54 g ● Fish were fed experimental diets and evaluated at 7[T1], 15[T2] and 21[T3] days
Tra catfish *(Pangasius hypophthalmus)*	(0, 62[1], 104[2], 237[3], 468[3], and 945[5] µg kg $^{-1}$ AFB$_1$) + (456 µg kg $^{-1}$AFB$_1$ + 1.5% B)	Gonçalves *et al.* 2018	n/a	n/a	=FI [T1-T3] ↓WG [T1-T3; 1-5] ↑FCR [T1-T3; 3-5] ↓SGR [T1-T3; 1-5]	↓Ery [T2-T3; 1-3] ↑Leu [T2-T3; 1-3] ↑HSI [T2-T3; 3] ↑ASI [T2-T3; 3] ↑AST [T2-T3; 3] ↑ALT [T2-T3; 2,3]	N y*[,1-3]	n/a	● Initial weight: 8.0±0.2 g; 12 weeks study; sampling at 4[T1], 8[T2] and 12[T3] weeks ● Tested a bentonite[B] (dioctahedral montmorillonite, Mycofix) as detoxifying strategy. ● Treatments[1,2] and [3] challenged with *Edwardsiella ictaluri** by bath.
Yellow catfish *(Pelteobagrus fulvidraco)*	(0, 200[1], 500[2], and 1,000[3] µg kg $^{-1}$ AFB$_1$) x with or without Mycofix	Wang *et al.* 2016	n/a	Y ↑GPT[3] ↑GOT[3]	Y ↑FCR[2,3] ↓FBW[3] ↓WG[3]	Y ↑A:G[3] ↓Lyz[3] ↓RBC[1-3] ↓TP[3] ↓bact.aw[3]	Y[1-3]	n/a	● Initial weight: 2.02 – 0.10 g, 12 weeks study ● Tested a bentonite (dioctahedral montmorillonite, Mycofix) as detoxifying strategy.

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Physiological parameters abbreviations: A:G= Albumin/globulin ratio; ADG= Absolute daily growth rate; AGR= Absolute growth rate; Alb= Albumin; ALP= Alkaline phosphatase; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; Bact.aw= Bactericidal activity; BactAT= Bacterial agglutination titter; CAT= Catalase; Cc.AK.aw= Cerebral cytosolic - adenylate kinase activity; Cc.CK.aw= Cerebral cytosolic - creatine kinase activity; Cc.PK.aw= Cerebral cytosolic - Pyruvate kinase activity; CF= Condition factor; CLRE= Crude lipid retention efficiency; Creat= Creatin; EROD= ethoxyresorufin O-deethylase; Ery= Erythrocyte count; FCR= Feed conversion ratio; FE= Feed Efficiency; HIS= hepatosomatic index; FER= Feed efficiency ratio; Fr= Feeding rate; Glob= Globulin; GOT= Glutamic-oxaloacetic transaminase; GR= Growth rate; GTP= Glutamic-pyruvic transaminase; HaeL= Haemolysine titre; Hb= haemoglobin; HbT= haemagglutination titre; Hct= haematocrit; HIS=

hepatosomatic index; Hta= hematopoietic tissue activity; IAPh= xAPh= Acid phosphatase, x=n, l, s; n=nuclear, l=lysosomal, s= soluble; Ig= immunoglobulin; KSI= kidney somatic index; Leuc= leucocytes; Lyph= Lymphocyte; Lyph Pr= Lymphocyte proliferation; Lyz= Plasma lysozyme concentration; Mcd.CK.aw= Mitochondria; creatine kinase activity; MESa= macrophage extracellular superoxide anion production; Mono= Monocytes; MR= Muscle ratio; nAPh= xAPh= Acid phosphatase, x=n, l, s; n=nuclear, l=lysosomal, s= soluble; Neut= Neutrophils; NO= nitric oxide; NTB= Nitroblue tetrazolium assay; PER= Protein efficiency ratio; Ph.aw= Phagocytic activity; Phl= Phagocytic index; Plat= Platelets; PPV= protein productive value (%); PRE= Crude protein retention efficiency; RBCs= Total erythrocyte count (red blood cells); ROS= Reactive oxygen species; sAPh= xAPh= Acid phosphatase, x=n, l, s; n=nuclear, l=lysosomal, s= soluble; SGR= Specific growth rate; SL= Standard length; SOD= Superoxide dismutase; SSi= spleen somatic index; Test= testosterone; TL= Total length; TP= Total protein; Try= Trypsin inhibition; UA= uric acid; Urea-N= Urea nitrogen; WBCs= Total leucocyte count (white blood cells); WG= Weight gain; XO.aw= xanthine oxidase (XO) activity; S/Pp.aw= Sodium-potassium pump (Na+, K+-ATPase)

Tissues or tissue related abbreviations: AbF= Abdominal fat; AChE= acetylcholinesterase adcyap1a= growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide (PACAP); AF= absolute brood amount; B= Brain; BBB= blood–brain barrier; BD= blindness; Bi= Bile; BL= Blood; Ca= Carcass; F= faeces; G= gills; GB= gall bladder; GI= Gastro intestinal track; GSI= gonadosomatic index; Haemr Ab= Haemoraghic Abdomen; Haemr Hd= Haemoraghic Head; Haemr SK= Haemoraghic skin; HK= head kidney; HP= Hepatopancreas; HT= hematopoietic tissue; HTi= Hepatic tumour incidence; I= intestine; K = Kidney; L = Liver; M = Muscle; Ood= oocyte diameter; RF= relative brood amount; RFs=rote fins; S= Stomach; SG= stomach gastric glands; SK = skin; Sp= Spleen; T= Testis; U= Urine;YS= yellow skin.

Table S1.5: Revision of literature on the effects of Aflatoxins on shrimp species.

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Black tiger shrimp *(Penaeus monodon)*	0, 5[1], 10[2] and 20[3] µg kg $^{-1}$AFB$_1$	Bintvihok *et al*. 2003	HP[1-3]	n/a	↓FBW[1-3;t2]	Y[1-3; t1-t2] ↓GOT ↓GTP N (Creat, Glu, Urea-N, TP, P)	Y[2,3;t1] Y[1-3;t2]	N	● 3.5 months old, 11 days study (samples at 8[t1] and 11[t2] days) ● AFB$_1$ was prepared from mouldy corn
Black tiger shrimp *(Penaeus monodon)*	0, 26.5[1], 52.3[2], 73.8[3], 100.8[4] and 202.8[5] µg kg $^{-1}$AFB$_1$	Bautista *et al*. 1994	Shdis[3-5] HP[3-5]	Y	↓WG[3-5] ↓SGR[3-5]	↑HaeI[1-5]		N	● Initial weight: 17.5 ± 0.6 g, 60 days study
Black tiger shrimp (Penaeus monodon)	0; 50[1]; 100[2]; 500[3]; 1,000[4]; 2,500[5] µg kg $^{-1}$ AFB$_1$	Boonyaratpalin *et al*. 2001	HP[3-5]	N	↓FBW[4,5] ↓WG[5]	N The, PO aw	Y[5]	n/a	● Study in juvenile stage = 0.7 g; 8 weeks trial ● Reddish discoloration dispersed over body and tail on 2500 µg kg $^{-1}$ AFB$_1$ treatment ● A bacterial suspension of *Vibrio harveyi* was injected into the tail muscle
Black tiger shrimp *(Penaeus monodon)*	0; 50[1]; 100[2]; 500[3]; 1,000[4]; 2,500[5] µg kg $^{-1}$ AFB$_1$	Boonyaratpalin *et al*. 2001	HP[2-5]	n/a	n/a	↑The[5,t1] ↑PO aw[3-5,t1] ↓Ca^{2+} [4,5,t2] ↓Cho[5,t2] N ALP, P	n/a	Head/ shell/ muscle (µg kg $^{-1}$) [1,t1]=2.6/13.0 [1,t2]=0.5/ 0.4 [2,t1]=3.5/ 14.2 [2,t2]=-/ 0.6 [3,t1]=9.1/ 10.6 [3,t2]=6.8/ 0.3 [4,t1]=2.3/8.4 [4,t2]=6.5/0.7 [5,t1]=3.9/7.4 [5,t2]=4.9/0.1	● Study in adult stage , Initial weight: 1.0-1.2 g; 8 weeks trial (sampling at 4[t1] and 6[t2] weeks)
Pacific white shrimp *(Litopenaeus vannamei)*	0; 50[1]; 250[2]; 1,500[3]; 3,000[4]; 15,000[5] µg kg $^{-1}$ AFB$_1$	Ostrowski-Meissner *et al*. 1995	HP[1-5] AG[1-5]	n/a	↓FBW[3-5] ↑FCR[1-5]	n/a	Y[5]	n/a	● Initial weight: 1.61±0.19 g, 21 days study ● Survival at 15 ppm = 0%

Pacific white shrimp *(Litopenaeus vannamei)*	15[1], 20[2], 60[3], 300[4], 400[5], 900[6] µg kg $^{-1}$ AFB_1	Ostrowski-Meissner *et al.* 1995	n/a	n/a	↓FBW[5,6] ↑FCR[5,6]	n/a	N	n/a	● Initial weight: 1.51±0.05 g, 8 weeks study ● Dry matter apparent digestibility coefficients, crude protein and digestible energy was decreased on 900 µg kg $^{-1}$ AFB_1 treatment
Pacific white shrimp *(Litopenaeus vannamei)*	0.1[1]; 1.0[2]; 10[3]; 100[4]; 1,000[5]; 10,000[6] ng ml^{-1} AFB_1	Burgos-Hernaandez *et al.* 2005	↓RTryp[6] ↓RColla [5] ↑RColla [6]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 5.5-6 g, 30 days study ● Study was undertaken to determine the in vitro effect of different mycotoxin concentrations on several parameters at hepatopancreas level.
Pacific white shrimp *(Litopenaeus vannamei)*	53,000[1]; 75,000[2]; 106,000[3]; 150,000[4]; 300,000[5] µg kg $^{-1}$ AFB_1	Wisemand *et al.* (1982)	HP[1-5] MO[1-5] HTo[1-5]	n/a	n/a	n/a	N	n/a	● Initial weight: 0.5 g, 28 days study
Pacific blue shrimp (*Penaeus stylirostri*)	0; 70,,000[1];115,000[2]; 160,000[3] µg kg $^{-1}$ AFB_1	Wisemand *et al.* (1982)	HP[1-3] MO[1-3] HTo[1-3]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 2.6±0.5 g, 10 days study ● Intravenously injection of AFB_1; ● 24-h LD_{50}=100,500 µg kg $^{-1}$ AFB_1 (95% confidence interval: 78.3 to 129.0) ● 96-h $LD5_{50}$= 49,500 µg kg $^{-1}$ AFB_1 (95% confidence interval: 29.8 to 82.3)
Black tiger shrimp *(Penaeus monodon)*	50[1]; 100[2]; 150[3]; 500[4]; 1,000[5]; 2,000[6] µg kg $^{-1}$ AFB_1	Gopinath *et al.* 2009	HP [T1, 1-6] HP [T2, 1-6] ↓Ls [T2, 1-6]	n/a	n/a	n/a	N	n/a	● Initial weight: 7.5±0.72 ● samplings at 4[T1] and 8 [T2] weeks
Pacific white shrimp *(Litopenaeus vannamei)*	0; 622[1]; 1,248[2]; 1, 769[3] µg kg $^{-1}$ AFB_1	Tapia-salazar *et al.* 2012	HP [1-3] ↓Ls [1-3] ↑HPs[2,3] ↑Tea[1-3]	n/a	↓WG[1-3] ↓FI[1-3] ↑FCR[3]	↓B-Cell.aw[1-3] ↓M.E-Cell.aw[2,3]	n/a	n/a	● Initial weight: 121±16 mg, 28 days study

Pacific white shrimp *(Litopenaeus vannamei)*	0; 10[1]; 20[2]; 40[3]; 60[4]; 120[5] µg kg $^{-1}$ AFB_1	Tapia-salazar *et al.* 2012	HP [1-5] =HPs[1-5] ↑Tea[2-5]	n/a	↓WG[4,5] ↓FI[1,2,5] ↑FCR[1]	↓B-Cell.aw[3-5] ↓M.E-Cell.aw[1,3,4]	n/a	n/a	● Initial weight: 614±7 mg, 64 days study
Pacific white shrimp *(Litopenaeus vannamei)*	16.9[1]; 38.1[2]; 54.9[3]; 107.6[4]; 461.8[5]; 1,092.1[6] µg kg $^{-1}$ AFB_1	Zeng *et al.* 2015	HP[4-6]	n/a	↓WG[2-6] ↓SGR[2-6] =FE, PER, HIS, CF	↑AST[2,5,6] ↑ALT[2,5,6] ↓Cho[5] =PO, T-AOC ↑GST[6]; ↓GST[6] =TP, Alb, TG	Y[2-6]	N	● Initial weight: 0.52 g; 8 weeks study ● Experimental control diet contaminated with 16.9[1] µg kg^{-1} AFB_1 ● "Based on this study, it was concluded that the AFB_1 level in Pacific white shrimp diet should be <38.1 µg kg $^{-1}$ AFB_1" Zeng et al. 2015
Pacific white shrimp *(Litopenaeus vannamei)*	0, 300[1], 400[2], 900[3] µg kg $^{-1}$ AFB_1	Divakaran and Tacon 2000	n/a	n/a	n/a	n/a	n/a	N	● Initial weight: 1.51±0.05 g; 8 weeks study
Pacific white shrimp *(Litopenaeus vannamei)*	(0 and 500[1] µg kg $^{-1}$ AFB_1) x (100 and 200mg kg $^{-1}$ Zn-CM)	Yu *et al.* 2018	HP[1] ↑HPs[1]	n/a	↓WG[1]	↑MDA[1] ↑GSH[1] ↑ALT[1] =SOD, CAT,GSH PX, iNOS, GR, NO, PO, AST	Y	N	● Initial weight: 1.08 g; 8 weeks study ● During acclimation (2weeks) shrimp were fed a commercial diet with 8.3 µg kg $^{-1}$ AFB_1
Pacific white shrimp *(Litopenaeus vannamei)*	0; 15,000 µg kg $^{-1}$ AFB_1	Zhao *et al.* 2017	HP[T3]	Y	n/a	↑SOD[T3] ↑GST[T3] ↑GPx[T3] ↑CAT[T3]	Y	n/a	● Initial weight: 2.40±0.13 g; 12 days study ● Shrimps were sampled at 1[T1], 2[T2], 8[T3] and 12[T4] days ●1,024 genes were differentially expressed in shrimp fed with AFB_1, being involved in functions, such as peroxidase metabolism, signal transduction, transcriptional control, apoptosis, proteolysis, endocytosis, and cell adhesion and cell junction
Pacific white shrimp *(Litopenaeus vannamei)*	0; 15,000 µg kg $^{-1}$ AFB_1	Zhao *et al.* 2018	HP[T3]	n/a	n/a	n/a	Y[T2, T3]	n/a	● Initial weight: 2.40±0.13 g; 12 days study ● Shrimps were sampled at 1[T1], 6[T2] and 12[T3] days ● Several genes were differentially expressed

									in shrimp fed AFB_1, being involved in metabolic functions, including the metabolism of pyrimidine, purine, mannose, arginine, proline, glycine, serine, galactose, sphingolipids, valine, leucine and isoleucine, and fatty acids
Pacific white shrimp *(Litopenaeus vannamei)*	0, 75[1] µg kg $^{-1}$ AF* and (75 µg kg $^{-1}$ AF) x AB or MS or MP *AFB_1 (60.7)+ AFB_2 (9.7) + AFG_1 (4.3 µg kg $^{-1}$)	Tapia-salazar *et al.* 2017	n/a	n/a	↓GR[1] ↓Nr[1]	n/a	N	n/a	● Initial weight: 210±4 mg ● AFB_1 levels achieve by formulating diets with contaminated corn (7,130 µg kg $^{-1}$; NuteK S.A) ● 3 aflatoxin binders that were tested: [AB]Aflabalan® (2 g kg $^{-1}$); [MS]Mycosorb® (2 g kg $^{-1}$) and [MP]Mycofix plus® (2.5 g kg $^{-1}$); (additional information about tested products at supplementary notes)
Pacific white shrimp *(Litopenaeus vannamei)*	0; 5,000 µg kg $^{-1}$ AFB_1	Wang *et al.* 2018	I	n/a	n/a	[HP ,I]↑GOT [HP ,I] ↑GPx [HP ,I] ↑CAT [HP ,I] ↓MDA	N	n/a	● Initial weight: 2.55 ± 0.08 g ● 30 days study with [I]Intestine and [HP]hepatopancreas samplings at: 3, 6, 12, 18, 24 and 30 days. ● AFB_1 could induce dysregulation of intestinal microbiota and damage of antioxidant system

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Physiological parameters abbreviations: Alb= Albumin; ALP= Alkaline phosphatase; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; B.cell.aw= B-cell activity; CAT= Catalase; CF= Condition factor; Cho= Cholestrol; Creat= Creatin; FBW= Final body weight; FCR= Feed conversion ratio; FE= Feed Efficiency; FI= Feed Intake; Glu= Glucose; GOT= Glutamic-oxaloacetic transaminase; GPx= Glutathione peroxidase; GR= Growth rate; GSHPX= glutathione peroxidase; GTP= Glutamic-pyruvic transaminase; HaeL= Haemolysine titre; HIS= hepatosomatic index; M.E-cell.aw= Mitotic E-cell activity; MDA= Malondialdehyde; NO= nitric oxide; NOS= nitric oxide synthase; NOS, namely eNOS (endo-thelial), nNOS (neuronal) and iNOS (inducible); Nr= Nitrogen retention; GST= Glutathione S transferase; P= Phosphorus; PER= Protein efficiency ratio; PO-aw= Phenoloxidase activity; RColla= Percentage of residual activity of collagen; RTryp= Percentage of residual activity of trypsin-like protease; SGR= Specific growth rate; SOD= Superoxide dismutase; T-AOC= total antioxidant capacity; TG= Triglyceride; The= Total haemocytes; TP= Total protein; Urea-N= Urea nitrogen; WG= Weight gain

Tissues or tissue related abbreviations: AG= Antennal gland; HPs= Hepatopancreatocyte sloughing; HTo=hematopoietic organs; I= intestine; Ls= Lipid storage; MO=Mandibular organ; Shdis=Shell discoloration; Tea= Tubular epithelial atrophy

Table S1.6: Revision of literature on the effects of Fumonisins or Moniliformin on fish species

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
African catfish *(Clarias gariepinus)*	0; 5,000[1]; 10,000[2]; 15,000[3] µg kg $^{-1}$	Gbore *et al*. 2010	n/a	n/a	↓FW[2,3] =WG, FI, FCR, SGR	↓Hct[1-3] ↓Ery[1-3] ↓Hb[1-3] ↑Leu[1-3] ↓MCV[1-3] ↓MHC[1-3] ↓TP[1-3] ↓Alb[1-3] ↓Glob[1-3] ↑A:G[1-3] ↑Gluc[2,3]	n/a	n/a	● Initial weight: 17.35±1.26 g ● FB_1 concentrations achieved by using *Fusarium*-culture maize. ● 6 weeks trial
Atlantic salmon *(Salmon salar)*	1,000; 5,000; 10,000 or 20,000 µg kg $^{-1}$	Garcia 2013	=Hist =LWC	n/a	=FI =FW =WG =FCR =SGR	n/a	N	n/a	● Initial weight: 31.8 ± 6.4 g, 10 weeks study ● mycotoxin levels on feeds were not confirmed/analysed or not reported
Channel catfish *(Ictalurus punctatus)*	300; 20,000[1], 80,000[2]; 320,000[3]; 720,000[4] µg kg $^{-1}$	Lumlertdacha *et al*. 1995	L[1-4;wk10]	n/a	↓WG[2-4;W2-W10] ↓WG[1;W6-W10]	↓Hct[2;W10] ↓RBC[2;W10] ↓WBC[2;W10]	Y[3,4;W4-W10]	n/a	● Initial weight: 1.2 g, 10 weeks study (sampling every week; wk1 – w10) ● Naturally contaminated corn containing 1,600 mg of FB_1 kg $^{-1}$
Channel catfish *(Ictalurus punctatus)*	300; 20,000[1]; 80,000[2]; 320,000[3]; 720,000[4] µg kg $^{-1}$	Lumlertdacha *et al*. 1995	L[1-4;wk14]	n/a	↓WG[3,4;W2-W14] ↓WG[2;W4-W14]	↓Hct[3,4;W14] ↓RBC[3,4;W14] ↓WBC[3,4;W14]	Y[3;W12,W14] Y[4;W14]	n/a	● Initial weight: 31 g, 14 weeks study (sampling every 2 week; wk2 – w14) ● Naturally contaminated corn containing 1,600 mg of FB_1 kg $^{-1}$ ● Clear age related effect
Channel catfish *(Ictalurus punctatus)*	300[1]; 2,500[2]; 5,000[3]; 10,000[4]; 20,000[5]; 40,000[6]; 80,000[7];	Goel *et al*. 1994		n/a	n/a	↑Sa/So; K[4-8] ↑Sa/So; Se[5-8] ↑Sa/So; L[6-8] ↑Sa/So; M[7] =Sa/So; B	n/a	n/a	● Initial weight: 20 g, 12 weeks study ● Diets contaminated by F. moniliforme corn culture material (1,600 mg FB_1 Kg $^{-1}$)

	240,000[8] µg kg $^{-1}$								
Channel catfish *(Ictalurus punctatus)*	700[1], 2,500[2]; 5,000[3]; 10,000[4]; 20,000[5]; 40,000[6]; 80,000[7]; 240,000[8] µg kg $^{-1}$	Li *et al*. 1994	L[6-8] B[6-8]	n/a	↓WG[6-8] ↓FI*[6] ↓FE[5,6,8]	↓Hct[7,8]	Y[5,6,8]	n/a	● Initial weight: 6.1 g, 12 weeks study (sampling every 2 week; wk2 – w14) ● Diets contaminated by *F. moniliforme* corn culture material (766 mg FB_1 Kg^{-1}) ●* Feed consumption on 80,000[7]; 240,000[8] µg FB_1 Kg $^{-1}$ was minimal FI was not calculated
Channel catfish *(Ictalurus punctatus)*	300[1]; 20,000[2]; 80,000[3]; 320,000[4]; 720,000[5] µg kg $^{-1}$	Lumlertdacha and Lovell, 1995	n/a	↓IgM[C;4,5]	↓WG[3;WK4-wk14] ↓↓WG[4,5;WK2-wk14]	n/a	Y[4,5;WK14] Y[C;4,5]	n/a	● Initial weight: 6.1 g, 12 weeks study (sampling every 2 week; wk2 – w14) ● Diets contaminated by *F. moniliforme* corn culture material (1605 mg FB_1 kg^{-1}) ● Treatments 3001; 20,000; 80,000 µg FB_1 Kg $^{-1}$ were challenged[C] (I.P.) with *Edwardsiella ictaluri*.
Clariid catfish *(Heterobranchus longifilis)*	2, 370; 14,680[1]; 24,740[2]; 43,040[3]; 82,770[4] µg kg $^{-1}$	Adeyemo *et al*. 2016	n/a	n/a	↓FW[T4; 1-4] ↓WG[T4; 1-4] ↓SGR[T4; 1-4] ↑FCR[T4; 1-4]	↑Ery[T2, T3, T4; 3,4] ↓Leuc[T1-T4; 31-4] ↓Hb[T1; 3] ↓Hb[T2; 4] ↓Hb[T4; 1-4] ↓PCV[T2-T4; 1-4]	n/a	n/a	● Initial weight: 151.64±2.11 g ● 56 days study, with samplings at 7[T1], 14[T2], 28[T3] and 56[T4] days. ● Control diet contained 2, 370 µg FB_1 kg $^{-1}$ feed
Common Carp *(Cyprinus carpio)*	0; 500[1]; 5,000[2] µg kg $^{-1}$	Pepeljnjak *et al*. 2003	Y Ds[2]	n/a	↓WG[1,2]	↑RBCs[1,2] ↓MCV[2] ↑RBCs[1,2] ↑Bir[1,2] ↑AST[1,2] ↑ALT[1,2] ↑TP[1,2] ↑Creat[1,2]	N	n/a	● Initial weight: 120-140 g, 42 days study ● Diets contaminated by *F. moniliforme* corn culture material ● No effects on: WBCs, Hb, Hct, MCH and MCHC
Common Carp *(Cyprinus carpio)*	0; 10,000[1]; 100,000[2] µg kg $^{-1}$	Petrinec *et al*. 2004	L[1,2] GB[1,2] HK[1,2] K[1,2]	n/a	N	n/a	N	n/a	● Initial weight: 15.87 g, 4 weeks study ● Petechial haemorrhages and oedema in kidney

			B[1,2]						
Common Carp *(Cyprinus carpio)*	0; 10,000[1]; 100,000[2] µg kg $^{-1}$	Kovacic *et al.* 2009	B[2]		↓WG[1,2]		N		● Initial weight: 70 - 131 g, 4 weeks study ●[1,2]FB_1 permeated the blood-brain barrier of young carp and had a toxic effect on neuronal cells.
Nile tilapia *(Oreochromis niloticus)*	0; 10,000[1]; 40,000[2]; 70,000[3]; 150,000[4] µg kg $^{-1}$	Tuan *et al.* 2003	N	n/a	↓WG[2,4;wk4-wk8] ↑FCR[2,4;wk8]	↓Hct[4;wk8] ↑Sa/So[4;wk8]	N	n/a	● Initial weight: 2.7 g, 8 weeks study (sampling every 2 week; wk2 – wk8) ● Diets contaminated by *F. moniliforme* corn culture material ● No histological changes on L, Sp, S, I, H,K
Nile tilapia *(Oreochromis niloticus)*	0; 20,000[1]; 40,000[2] and 60,000[3] µg kg $^{-1}$ $FB_1 + FB_2$	Claudino-silva *et al.* 2018	↓[L]IGF-1[1-3] ↓[L]GHR[1-3]	n/a	↓WG[1-3]	n/a	Y[1-3]	n/a	● Initial weight: 2.64±0.06 g ● 30 days study, with samplings at 15[T1] and 30[T2] days
Rainbow trout *(Oncorhynchus mykiss)*	0; 600[1], 20,000[2]; 63,000[3] µg kg $^{-1}$	Meredith *et al.* 1998 and Riley *et al* 2001	n/a	n/a	n/a	↑Sa/So L[2,3] K[2-3] Se[2-3]	n/a	n/a	● Initial weight: 15-25 g, 1 week study
Rainbow trout *(Oncorhynchus mykiss)*	0; 66,000[1]; 139,000[2]; 281,000[3] µg kg $^{-1}$	Meredith *et al.* 1998 Study II	n/a	n/a	n/a	↑Sa/So K[1-3] L[1-3]	n/a	n/a	● Initial weight: 15-25 g, 5 days study
Moniliformin									
Nile tilapia *(Oreochromis niloticus)*	0; 10,000[1]; 40,000[2]; 70,000[3]; 150,000[4] µg kg $^{-1}$ MON	Tuan *et al.* 2003	N	n/a	↓WG[3,4;wk4-wk8] ↑FCR[3,4;wk8]	↓Hct[3,4;wk8] ↓Pyr[1,4;wk8]	N	n/a	● Initial weight: 2.7 g, 8 weeks study (sampling every 2 week; wk2 – wk8) ● Diets contaminated by *F. proleferatum* corn culture material ● No histological changes on L, Sp, S, I, H,K

Zebrafish *(Danio rerio)*	0; 18[1]; 90[2]; 450[3]; 900[4]; 1,350[5] and 1,800[6] µg L $^{-1}$ MON Study 3 100[7]; 316[8]; 1,000[9]; 3,160[10]; 10,000[11] µg L $^{-1}$ MON	Gonçalves *et al*. 2018	↓Cell.v-Prolif[9-11] ↓Cell.v-Cytotox[9-11]	n/a	n/a	n/a	Y[4-6]	n/a	● Zebrafish (AB wild-type strain) ● A negative control (H2O) and a positive control (calcitriol) was used Study 1 – Operculum mineralization ● Larvae were exposed for 3 days Study 2 – Effects on deformities ● Larvae were exposed until 20 dpf Study 3 – Cytotoxicity, proliferative and mineralogenic effects *in vitro* ● VSa13 cell line—a mineralogenic cell type derived from gilthead seabream *S. aurata* vertebra was used

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Physiological parameters abbreviations: A:G= Albumin/globulin ratio; Alb= Albumin; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; Bir= Bilirubin; Cell.v= Cell viability (Cytox= Cytotoxicity or Prolif= Proliferative); Creat= Creatin; Ery=Erythrocyte count; FCR= Feed conversion ratio; FE= Feed efficiency; FI= Feed intake; FW= Final weight; Glob= Globulin; Glu= Glucose; Hb=haemoglobin; Hct=haematocrit; Ig= immunoglobulin; Leuc= leucocytes; MCV= mean erythrocyte volume; MHC= major histocompatibility complex; PCV= Packed-cell volume; Pyr= Serum pyruvate; RBCs= Total erythrocyte count (red blood cells); Sa/So= sphinganine/ sphingosine; TP= Total protein; WBCs= Total leucocyte count (white blood cells); WG= Weight gain

Tissues or tissue related abbreviations: B= Brain; Ds= Dilation of sinusoids; GB= gall bladder; Hist= Histology; Hist= Histology; HK= head kidney; K= Kidney; L= Liver; LWC= Liver water content; Se= Serum

Gene abbreviation: *GHR*= growth hormone receptor; *IGF-1*= insulin growth factor 1

Table S1.7: Revision of literature on the effects of Fumonisins on shrimp species

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Pacific white shrimp *(Litopenaeus vannamei)*	0; 200[1]; 600[2]; 2000[3] µg kg $^{-1}$	García-Morales *et al*. 2015	Mms[1-3] Pd[1-3]	n/a	↓FW[2,3]	n/a	N	n/a	● Initial weight: 5.5-5.7 g, 30 days study Soluble muscle protein concentration decreased, and changes in myosin thermodynamic properties were observed in shrimp after 30 days of exposure to FB .Marked histological changes in tissue of shrimp fed a diet containing FB_1 at 2.0 µg g $^{-1}$ were also observed. Shrimp fed diets containing more than 0.6 µg g $^{-1}$ FB showed greater decrease in shear forces after12 days of ice storage.
Pacific white shrimp *(Litopenaeus vannamei)*	0.25[1]; 0.50[2]; 1.0[3]; 2.0[4] µg ml $^{-1}$	Burgos-Hernaandez *et al*. 2005	↓RTryp[1-4] ↓RColla[1-3] ↑RColla[4]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 5.5-6 g, 30 days study ● Study was undertaken to determine the in vitro effect of different mycotoxin concentrations on several parameters at hepatopancreas level.
Pacific white shrimp *(Litopenaeus vannamei)*	500[1]; 750[2]; 1,000[3] µg kg $^{-1}$	Mexía-salazar *et al*. 2008	HP[1-3] M[1-3]	↓Pro-PO[1-3] ↓Phose[1-3] ↓Supa[1-3] ↓THC[1-3]	n/a	n/a	n/a	n/a	● Initial weight: 5-6 g, 18 days study ● Changes in both, electrophoretic patterns and thermodynamic properties of myosin extracted from shrimp exposed to FB_1

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.
Physiological parameters abbreviations: FW= Final weight; Phose=Phenoloxidase; Pro-PO= Prophenoloxidase; Supa= Superoxide anion; THC= Total haemocyte counts
Tissues or tissue related abbreviations: HP= Hepatopancreas; M= Muscle; Mms= Muscle myofibrillar structure; Pd= Protein degradation; RColla= Percentage of residual activity of collagen; RTryp= Percentage of residual activity of trypsin-like protease

Table S1.8: Revision of literature on the effects of Ochratoxins on fish species

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Atlantic salmon *(Salmon salar)*	0, 800[1] and 2,400[2] µg kg $^{-1}$	Bernhoft *et al.* 2017	n/a	n/a	↓FI[1,2]	n/a	n/a	L/M/K/SK (µg kg $^{-1}$) [1,t1]=1.86/<LOQ/n.s./n.s. [1,t2]=1.53/<LOQ/n.s./n.s. [1,t3]=1.01/<LOQ/n.s./n.s. [2,t1]=4.81/ <LOQ/n.s./n.s. [2,t2]=3.27/ <LOQ /n.s./n.s. [2,t3]=2.61/ <LOQ/n.s./n.s.	● Initial weight: 58 g, 8 weeks study ● Sampling at 3[t1], 6[t2] and 8[t3] weeks
Channel catfish *(Ictalurus punctatus)*	0; 2,000[1] and 4,000[2] µg OTA kg $^{-1}$	Manning *et al.* 2005	n/a	n/a	↓WG[1,2]	n/a	n/a	↑Mort[C; 1,2]	● Initial weight: 6.4, 6 weeks feeding study ● Challenge[C] (by bath) with *Edwardsiella ictaluri* for 21 days (estimated 2.25 x 10^6 colony forming units per milliliter of water)
Channel catfish *(Ictalurus punctatus)*	0; 500[1]; 1,000[2]; 2,000[3]; 4,000[4]; 8,000[5] µg OTA kg $^{-1}$	Manning *et al.* 2003	L, K ↑MMC	n/a	↓WG[2-5,WK2,8] ↓WG[3-5,WK4,6] ↓FCR[4,5]	↓Htc[5] ↑WBCs	Y[5]	n/a	● Initial weight: 6.1 g, 8 weeks study (sampling every 2 week; wk2 – wk8) ● Diets contaminated by *Aspergillus ochraceus* culture material
European seabass *(Dicentrarchus labrax)*	0, 50, 100, 150, 200, 250, 300, 350 and 400 µg kg $^{-1}$	El-Sayed *et al.* 2009	n/a	n/a	n/a	n/a	µg kg $^{-1}$ LC_1=146.2 LC_5=176.3 LC_{10}=195.7 LC_{15}=208.3 LC_{50}=277.0 LC_{85}=368.7 LC_{90}=393.2 LC_{95}=434.4	n/a	● Initial weight: 40±2 g, sampling at 24, 48, 72 and 96 h, ● OTA-contaminated feed was administered to individual sea bass once daily by oral gavage

							LC_{99}=523.7		
Rainbow trout (*Salmo gairdneri*)	OTA: 2,000[1]; 3,000[2]; 4,000[3]; 5,000[4]; 6,000[5] and 8,000[6] µg OTA per kg $^{-1}$ body weight OTB: 16,700[7]; 33,300[8] and 66,700[9] µg OTB per kg $^{-1}$ body weight OTα: 7,000[10], 14,000[11] and 28,000[12] µg OTα per kg $^{-1}$ body weight OTβ: 6,700[13], 13,300[14] and 26,700[15] µg OTβ per kg $^{-1}$ body weight	Doster *et al.* 1972	Haemr_L [1-6] K [1-6] L [1-6]	n/a	n/a	n/a	OTA: LC_{50}=4,670 = OTB, OTα, OTβ	n/a	● Initial weight: 30 g, 10 days study ● Diets contaminated by *Aspergillus ochraceus* culture material from wheat ● single intra-peritoneal dose of one of the four ochratoxins
Zebrafish *(Danio rerio)*	[5,000 µg L $^{-1}$ to 0.039 µg L $^{-1}$] 160[1]; 310[2], 630[3]; 1,250[4]; 2,500[5]; 5,000[6] µg L $^{-1}$	Tschirren *et al.* 2018	↑ED [t1; 5,6] ↑ED [t2; 4-6] ↑ED [t3; 3-5] ↑ED [t4; 2,3] ↓Hr [t2; 3-6] ↓Hr [t3; 3-6]	n/a	n/a	↑Ox.S [t3; 1,2]	↑Mort. [t2; 6] ↑Mort. [t2; 5,6] ↑Mort. [t3; 6 (100%)] ↑Mort. [t4; 2,3 /4-6-100%] ↓Hatch.S [t3,t4; 1-3] Hatch.S= 0% [t3,t4; 4-6] LC_{50} [t1]= 2,650 µg L $^{-1}$ EC_{50} [t1]= 24,220 µg L $^{-1}$ LC_{50} [t2]= 730 µg L $^{-1}$ EC_{50} [t2]= 2,570 µg L $^{-1}$ LC_{50} [t4]= 290 µg L $^{-1}$	n/a	● Eggs were incubated with different ochratoxin concentrations at 3 h post fertilization ● Evaluations were made at 24[t1], 48[t2], 72[t3] and 96[t4] h

							EC_{50} [t4]= 360 µg L^{-1}		
Zebrafish *(Danio rerio)*	OTA: 0.05[1], 0.1[2], 0.25[3], 0.5[4], 1[5], 2[6] and 2.5[7] µM OTα: 0.1[8], 0.25[9], 0.5[10], 1[11] and 2.5[12] µM	Haq *et al*. 2016	↑DEF[T1; 5,7] ↑DEF[T2; 4-7] ↑DEF[T3; 2-7] ↑DEF[T4; 1-7]	n/a	n/a	n/a	↑Mort. [t2; 5-7] ↑Mort. [t3; 4-7]	n/a	● Zebrafish was exposed from ≤2 hours post-fertilization [hpf], 4–32 cell stage until 5 days post-fertilization (dpf) ● samplings at 1[T1], 2[T2], 4[T3] and 5[T4] days post-fertilisation

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.
Physiological parameters abbreviations: WG= Weight gain; FCR= Feed conversion ratio; Hct=haematocrit; WBCs= Total leucocyte count (white blood cells); LCxx= Lethal concentration at xx dosage; Mort= Mortality; Hatch.S= Hatching success; ECxx= effective concentration
Tissues or tissue related abbreviations: L= Liver; K= Kidney; MMC= melanomacrophage centers; Haemr= Haemoraghic; ED= damage to the embryos; Hr= Heart rate; DEF= deformation

Table S1.9: Revision of literature on the effects of Ochratoxins on shrimp species

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Black tiger shrimp *(Penaeus monodon)*	100[1]; 200[2] and 1,000[3] µg kg^{-1}	Supamattaya *et al.* 2005	N	Y[3]	N	↓ALP[1-3] ↓SGOT[1,2] ↓SGPT[2] ↓PO_aw[3]	N	< 44,000 µg kg^{-1} (LOD)	● Initial weight: 2 g; 8 weeks study ● No differences on THC or Ca^{2+} levels ● No differences in tissues: G, AG, HP, HT,

Physiological parameters abbreviations: ALP= Alkaline phosphatase; SGOT= Glutamic-oxaloacetic transaminase; SGPT= Glutamic-pyruvic transaminase; PO-aw= Phenoloxidase activity

Table S1.10: Revision of literature on the effects of Zearalenone and its metabolites in aquaculture species

Species	Tested dosage	Reference	Tissue alterations	Immuno suppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Common Carp *(Cyprinus carpio)*	0; 332[1]; 621[2] and 797[3] µg kg $^{-1}$	Pietsch *et al*. 2015	n/a		=FW =WG =SGR =FCR =CF	↓Mono[2,3] ↓gran[2,3] ↓MNery[1-3] N =Leuc; Lyph; Hct; Hg; Vtg	n/a	M[1-3] ZEN=0.13[1]/0.22[2]/0.15[3] ng d $^{-1}$ dry weight α-ZEN=0.11[1]/0.16[2]/0.05[3] ng d $^{-1}$ dry weight	● Raised from egg used with 12-16 cm in length, ● 4 weeks study ● α-ZEN were not detectable after recover period (2 weeks) and ZEN were detected at 0.03 ng d $^{-1}$ dry weight for all treatments
Common Carp *(Cyprinus carpio)*	0; 332[1]; 621[2]and 797[3] µg kg $^{-1}$	Pietsch *et al*. 2016	LDH=↑K[2], ↓G[3],M[1] MDA=↓L[3]; ↓G[1]; ↑K[1]		n/a	↓LDH[3] ↑TP[3] ↑O_2 C[2,3]	n/a	n/a	● Raised from egg used with 12-16 cm in length, 4 weeks study ● Measured ADH, ALT, AST, LDH, MDA on serum and tissues (K, HK, Spleen, L, I, G, M)
Common Carp *(Cyprinus carpio)*	0; 332[1]; 621[2]and 797[3] µg kg $^{-1}$	Pietsch *et al*. 2017	↓*vtg*[RP1,3] ↑*tf*[RP1-3]	↓*IL-1β*[RP, 3] ↓*IL-8*[RP, 3] ↓*tgf-β*[1-3;RP 1-3] ↓*il-10*[RP, 3] ↓*arg-1*[1;RP1] ↓*arg-2*[RP1-3]		↓*Mn-sod*[RP2] ↓*cat*[RP1-3] ↓*v-atpase*[1-3; RP1-3]			● Initial weight: 24.9 ± 0.4 ● Four weeks trial being fed ZEN with additional 2 weeks of feeding uncontaminated diet – recovery period[RP] ● "this study suggests that the current maximum allowable levels in compound feed are too high to prevent damage to fish"
Rainbow trout *(Oncorhynchus mykiss)*	0; 2,000[1] µg kg $^{-1}$	(Woźny *et al.*, 2019)	K[1] HK[1] Sp[1]	Y[1]	↓FCR[1] ↑SGR[1]	↓A:G[1] ↑Hb[1] ↓Lymph[1] ↓Basph[1] ↑Throb[1]	N	I ZEN= x̃ =60.5 µg kg $^{-1}$ α-ZEL= x̃ =18.1 µg kg $^{-1}$ β-ZEN= x̃ =14.8 µg kg $^{-1}$	● Raised from egg up to commercial size; 96 weeks study. ● Trout's density adjusted at 29, 60 and 79 weeks ● Fish that had been exposed for 72 weeks, the mycotoxin's residuals

							L ZEN= x̃~0 µg kg $^{-1}$ α-ZEL= x̃ =28.2 µg kg $^{-1}$ β-ZEN= x̃ =6.2 µg kg $^{-1}$ M = 0 µg kg $^{-1}$	were not transferred to the fishes' muscles ●↑ Vulnerability to *Tetracapsuloides bryosalmonae*
Rainbow trout *(Oncorhynchus mykiss)*	1,810 µg kg $^{-1}$	Woźny *et al*. 2015	L	N	N =Glu =TP =Alb =AST =ALP =TAG	N	Y I ZEN = 732.2 µg kg $^{-1}$ α-ZEN=10.7 µg kg $^{-1}$ L=residual ZEN and α-ZEN in all sampled fish	● Initial weight: 250 g, all females; 71 days study ● Some of animals were identified as males ● ZEN was detected (<5.0 µg·kg $^{-1}$) in all females ovaries
Rainbow trout *(Oncorhynchus mykiss)*	1,000 µg ZEN per k $^{-1}$ of body mass	Woźny *et al*. 2017	n/a	n/a	n/a	n/a	Y (µg kg $^{-1}$) ZEN/ α-ZEN /β-ZEN I ^{48h}=~1500/~600 I ^{96h}=~1500/~900 L ^{48h}=~700/~100/~500 L ^{96h}=<200/<20/~0 O ^{48h} =321/~100 O ^{96h}=<100/<100 Oo ^{48h} =~25/~10 Oo ^{96h}=<5/<5 Plasma ^{48h} =~10/~5 Plasma ^{96h} =~0/~0 M ^{48h} =~5/~5 M ^{96h} =~3/~3	● Initial weight: 1274±162 g, all mature females ● Objective was to study the ZEN carry-over to eggs ● Administration on ZEN – Oral (bolus) ● Sampling periods: 2, 6, 12, 24, 48, 72, 96h ● Verified the presence of ZEN and α-ZEN in commercial fish roe ● "Contamination of fish roe with ZEN residuals is unlikely to pose a health risk to consumers, but their potential to transfer to somatic cells in fish ovaries may be of concern for aquaculture"
Rainbow trout *(Oncorhynchus mykiss)*	10,000 µg ZEN kg $^{-1}$ of body mass	Woźny *et al*. 2012	n/a	n/a	↑Bct [Fe]:↓L $^{24\text{-}168h}$; ↓O $^{24\text{-}168h}$ =AlT =AST =Glu	n/a	n/a	● Initial weight: 53.3±5.3 g, all mature females; ● Injected intraperitoneally with ZEN ● Sampling periods:24, 72, 168h

Rainbow trout *(Oncorhynchus mykiss)*	10,000 µg kg $^{-1}$ ZEN or one of the other compunds[#1] *Only effect of ZEN will be characterized	Woźny *et al*. 2008	n/a	n/a	n/a	↑*ERα* ↑*Zr.P* ↑*CYP1A*	n/a	n/a	● Initial weight: 50.3±3.4 g; ● injected intraperitoneally with one of the target compounds[#1] cyclopenta[c]phenanthrene (CP[c]Ph); its derivatives, 5A-CP[c]Ph; 5A6M-CP[c]Ph; 5A9M-CP[c]Ph; B[c]Ph, a structurally similar polycyclic aromatic hydrocarbon; B[a]P, a model CYP1A inducer; and zearalenone (ZEA), naturally occurring ligand for Estrogen receptor
[RT]**Rainbow trout** *(Oncorhynchus mykiss)* + [AS]**Atlantic salmon** *(Salmon salar)*	[AS]Positive control= 5,000 µg estradiol-17β kg $^{-1}$ BW; [AS,1]1000 µg kg $^{-1}$ α-ZEL [AS,2]1000 µg kg $^{-1}$ β –ZEL [AS,3]10,000 µg kg $^{-1}$ α-ZEL [AS,4]10,000 µg kg $^{-1}$ β –ZEL [AS,5]1000 µg kg $^{-1}$ ZEN [AS,6]10,000 µg kg $^{-1}$ ZEN	Arukwe *et al*. 1999	n/a	n/a	n/a	↑Zr.P[AS;1,3,5 6] ↑*Vtg*[AS;3, 5, 6]	n/a	n/a	First study ● Initial weight: 400-600 g rainbow trout; ● Study the competition for estrogen receptor (in vitro binding affinity) Second study ● 55±8g Atlantic salmon;7 days study ● Injected intraperitoneally with α\β-ZEN ● estrogenic potency in both in vitro receptor competitive binding and in vivo induction of Vtg and Zr-proteins levels is: α-ZEL >ZEN>β- ZEL
Zebrafish *(Danio rerio)*	0, 0.5[1], 1[2], 5[3] and 10[4] µg l $^{-1}$	Sellamani *et al*. 2018	↓GSI[3,4] O[2-4] ↑O-*Caspase-3*[3,4] ↑L-*ERα*[3,4] ↑L-*Vtg*[3,4] Oo.a[3,4] Oo.d[3,4]	n/a	n/a	n/a	n/a	n/a	● Wild-type TL (Tupfel Longfin) zebrafish ● Fish exposed to ZEN by bath in a semi-static system (100% renewal every 48h); during 21 days

Zebrafish *(Danio rerio)*	1[1] ng L $^{-1}$, 0.1[2], 1[3], 5[4], 10[5], 25[6], 50[7], 100[8], 50[9], 500[10], 750[11], 1000[12], 1250[13], 1500[14], 1750[15], 2000[16], 3000[17], 4000[18], 5000[19] µg L $^{-1}$	Bakos *et al.* 2013	Edema [T1]=1500 [T2]=1000 [T3]=100 [T4]=100 [T5]=100 Dorsal body axis curvature [T2]=500 [T3]=500 [T4]=750 [T5]=750 Reduced pigmentation [T3]=750 [T4]=50 [T5]=50 Unhatched larvae [T3]=1000		n/a	n/a	LC_{50}= 2854 µg L $^{-1}$ LC_{50}= 2068 µg L $^{-1}$ LC_{50}= 1299 µg L $^{-1}$ LC_{50}= 1100 µg L $^{-1}$ LC_{50}= 893 µg L $^{-1}$	n/a	● Embryo test ● From 1 h post fertilization until 5 days post Fertilization ● Sampligs at: 24[T1], 48[T2], 72[T3], 96[T4], 120[T5] hpf ● Exposure by bath
Zebrafish *(Danio rerio)*	0.1[1], 10[2], 1000[3] µg L $^{-1}$	Bakos *et al.* 2013	↑Vtg[3] ↑*vtg-1* mRNA[2,3]		n/a	n/a	n/a	n/a	● Adult fish; 21 days study ● Exposure by bath ● Sexually mature males
Zebrafish *(Danio rerio)*	0, 100[1], 320[2], 1000[3] and 3200[4] ng L $^{-1}$	Schwartz *et al.*, 2010	↑Vtg[3,4] ↓SF[PE,E] ↓CS[1,2,4;PE,E] ↓Fecund[2-4;PE,E] = gonad morphology		=BW =BL	n/a	N	n/a	● Spawning groups (two female and four male) ● Pre-exposure[PE] spawning period of 21 days and 21 days exposure[E] during spawning ● Spawned eggs were removed from exposure tanks and examined
Zebrafish *(Danio rerio)*	0, 0.1[1], 0.32[2] and 1[3] µg L $^{-1}$	Schwartz *et al.* 2013	n/a	n/a	↑Lg[H] ↑LW[H] ↑CF[Hc; H] ↓♂/♀[M,H] ↑RFecund[H] =RSF ↑VTGc[H]	n/a	n/a	n/a	● Whole life cycle (eggs to adult; 224 days) ● ZEN supply solution was by means of a computer-controlled dispenser at a rate of 600, 1920, and 6000 µL h $^{-1}$ giving nominal exposure concentrations of 0.1, 0.32 and 1 µg L $^{-1}$, respectively.

									● Experimental groups were divided as: Control[C]; Low (0.1 µg L $^{-1}$) during Reproduction (F0), Juvenile (F1) and reproduction (F1) –L[L] or Lc[Lc] (recovery during juvenile phase): Medium (0.32 µg L $^{-1}$; M[M] or Mc[Mc]) and High (1 µg L $^{-1}$; H[H] or Hc[Hc]).
T2 and HT-2									
Channel catfish *(Ictalurus punctatus)*	0; 1,000[1] and 2,000[2] µg T-2 kg $^{-1}$	Manning *et al*. 2005	n/a	n/a	↓WG[1,2]	n/a	n/a	↑Mort[C; 1,2]	● Initial weight: 6.4, 6 weeks feeding study ● Challenge[C] (by bath) with *Edwardsiella ictaluri* for 21 days (estimated 2.25 x 10^6 colony forming units per milliliter of water)
Common carp *(Cyprinus carpio)*	0; 4,110 µg T-2 kg $^{-1}$ + 490 µg HT-2 kg $^{-1}$	Pelyhe *et al*., 2016	HP =MDA; GSH; Gpx ↑GPx[t3]	n/a	n/a	↓GSH[t3] ↓GSH[t2] ↓*gpx4a*[t2,t4] ↑*gpx4a*[t3] ↑*Gpx4b*[t1-t3]	Y	n/a	● Initial weight: 23.26±6.86, 4 weeks study ● Control diet contaminated with: T-2, <20 µg kg $^{-1}$; HT-2,<20 µg kg $^{-1}$; DON, <20 µg kg $^{-1}$; and 15-acetyl DON,<20 µg kg $^{-1}$ ● Sampling at 7[t1], 14[t2], 21[t3], 28[t4]

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Physiological parameters abbreviations: A:G= Albumin/globulin ratio; Alb= Albumin; ALP= Alkaline phosphatase; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; Basph= Basophiles; Bct= Blood clotting time; BL= Body length; CAT= Catalase; CF= Condition factor; CF= Condition factor; CS= Clutch size; F= relative spawning frequency; FCR= Feed conversion ratio; Fecund= Fecundity; FW= Final weight; Glu= Glucose; Gran= Granulocytes; Hb=haemoglobin; Hct=haematocrit; IL = interleukin; LDH= lactate dehydrogenase; Leuc= leucocytes; Lg= Length; LW= Liver weight; Lyph= Lymphocyte; MDA= Malondialdehyde; MNery= Micronuclei (MN) in erythrocytes; Mono= Monocytes; O_2 C= Oxygen consume; RFecund= relative fecundity; SF= Spawning frequency; SGR= Specific growth rate; TAG= triacylglycerides; Throb= Thrombocytes; TP= Total protein; TP= Total protein; VTGc= Vitellogenin concentration; WG= Weight gain; Zr.P= zona radiata protein

Tissues or tissue related abbreviations: Erα= Estrogen receptor α; G= Gills; GSI = gonadosomatic index; I= Intestines; K= Kidney; L= Liver; M= Muscle; O= Ovary; Oo.a= oocyte atresia; Oo.d= oocyte membrane detachment; Vtg= Vitellogenin

Gene abbreviation: arg= arginase; CYP1A= Cytochrome P 1A; Erα= Estrogen receptor α; mn-sod= manganese-dependent enzyme superoxide dismutase; tf = iron-binding protein transferrin; v-atp-ase= v-type H+ ATPase

Table S1.11: Revision of literature on the effects of Zearalenone on shrimp species

Species	Tested dosage	Reference	Tissue alterations	Immunosuppressive	Performance alterations	Hematopoietic alterations	Increased Mortality	Residues in tissues	OBS.
Black tiger shrimp *(Penaeus monodon)*	0; 100[1]; 500[2] and 1,000[3] µg kg $^{-1}$	Bundit *et al*. 2006	HP[T1; 3]; HT[T1; 3]; Lyph[T1, 3] HP[T2; 3]; HT[T2; 3];	n/a	n/a	n/a	n/a	n/a	● Initial weight: 2 g; 8[T1] and 10[T1] weeks study

Tissues or tissue related abbreviations: HP= hepatopancreas; HT= haematopoietic tissue; Lyph = lymphocites

Table S1.12: Revision of literature on the effects of mycotoxin multi-exposure on fish and shrimp species

Species	Mycotoxin contamination (µg kg $^{-1}$)	Reference	Tissue alterations	Immuno suppressive	Growth performance alterations	Hematopoietic alterations	Increased mortality	Residues in tissues	OBS.
African catfish *(Clarias gariepinus)*	[C]2 + 3,000 AFB_1/FB_1 [1]7.3+15,000 AFB_1/FB_1 [2]17.6+24,500 AFB_1/FB_1 [3]48+43,000 AFB_1/FB_1 [4]93+83,000 AFB_1/FB_1	Adeyemo *et al.* 2018	n/a	n/a	↓FW [T4; 1-4] ↓WG [T4; 1-4] ↓SGR [T4; 1-4] ↑FCR [T4; 1-4]	↓Ery [T1; 2-4] ↓Ery [T2-T4; 1-4] ↓Hb [T1; 2-4] ↓Hb [T2; 1-4] ↓Hb [T3; 3,4] ↓Hb [T4; 1-4] ↓PCV [T1; 2-4] ↓PCV [T2-T4; 1-4] ↓Leuc [T1-T4; 1-4]	N		● Initial body weight of 15 g up to 296 – 320g ● 56 days study; samplings at 7[T1], 14[T2], 28[T3] and 56[T4] days ● Control[C] diet contaminated by AFB[1] and FB[1]
Atlantic salmon *(Salmon salar)*	DON= [19.4-22.4] µg kg $^{-1}$ T2= [0.1] µg kg $^{-1}$ FUM=[112-754] µg kg $^{-1}$	Nacher-Mestre *et al.* 2015	n/a	n/a	n/a	n/a	n/a	N	● Initial weight: 228±5 g, 6 months study ● The study surveyed commercially available plant ingredients (19) and PAP (19) for 18 mycotoxins
Channel Catfish *(Ictalurus punctatus)*	MON= 0; 20,000[1]; 40,000[2]; 60,000[3] and 120,000[4] µg kg $^{-1}$ FB_1= 0; 20,000[5] and 40,000[6] µg kg $^{-1}$ [7]MON:FB_1= 40,000:20,000 µg kg $^{-1}$ [8]MON:FB_1= 20,000:40,000 µg kg $^{-1}$	Yildrim *et al.* 2000	↓Hep.N [3,4/ 7,8]	n/a	↓FW[1-4/ 5,6/ 7,8] ↓FI[1-4/ 5,6/ 7,8] ↑FCR[3,4/ 5,6/ 7,8]	↓Hct[3,4/ 6/ 8] ↑Pyr[3/ 8] ↑Sa/So[5,6/ 7,8]	N	n/a	● Initial weight: 1 g ● MON concentrations achieved by using *Fusarium proliferatum* culture material. ● FB_1 concentrations achieved by using *Fusarium moniliforme* culture material.
Common carp *(Cyprinus carpio)*	AFB_1=0[1], 0.01[2] and 0.02[3] µg L $^{-1}$ DON= 0[4], 0.25[5], 0.5[6] µg L $^{-1}$ AFB_1:DON = 0.01:0.25[7]; 0.01:0.5[8]; 0.02:0.25[9]; 0.02:0.5[10]	He *et al.* 2010	↑IR*[2,3,5,6] ↑↑IR*[7-10] ↑AST[2,3,5,6, 7-10, T1] ↑ALT[2,3,5,6, 7-10, T1-T3] ↑LDH[2,3,5,6, 7-10, T1]						● *Primary hepatocytes were used in this study ● Enzyme Activity of AST, ALT and LDH in Cell Supernatant sampled at 4[T1], 8[T2] and 16h[T3]

Common carp *(Cyprinus carpio)*	C^{C}=15 + 22 AFB_1/OTA $^{P0.2\%}$=12 + 14 AFB_1/OTA $^{P0.4\%}$=9 + 6 AFB_1/OTA $^{B0.15\%}$ =21+ 13 AFB_1/OTA $^{B0.25\%}$ =21+ 15 AFB_1/OTA (µg kg $^{-1}$)	Agouz and Anwer (2011)	n/a	n/a	↓FW^{C} ↓TWG^{C} ↓SGR^{C} ↑FI^{C} ↑FCR^{C} ↓PER^{C}	↓Htc^{C} ↑WBCs^{C} ↑RBCs^{C} ↓Hb^{C}	Y^{C}	n/a	● Initial weight: 15 g, 8 weeks study (sampling every 2 week; wk2 – wk8) ● Tested a synthetic probiotic composed by: Bacillus subtilis, proteolytic, lipolytic amylolytic and cell separating enzymes, gemanium and organic selenium (inclusion of 0.2%$^{P0.2\%}$ and 0.4% $^{P0.4\%}$). ● Tested a commercial smectite clay composed by sodium/calcium aluminosilicate (HSCAS; inclusion of 0.15%$^{B0.15\%}$ and 0.25% $^{B0.25\%}$) ● Control treatment contains the highest values of AF and OTA; evaluation refers only to control compared to remain treatments
Gilthead seabream *(Sparus aurata)*	Diet 1 = 79.2 µg kg $^{-1}$ DON + 8.1 µg kg $^{-1}$ 15-AcDON Diet 2= 53.5 DON + 13.6 µg kg $^{-1}$ 15-AcDON + 6.4 µg kg $^{-1}$ FUM	Nacher-Mestre *et al.* 2015	n/a	n/a	n/a	n/a	n/a	N	● Initial weight:15 g, 8 months study ● The study surveyed commercially available plant ingredients (PP) (#19) and processed animal proteins (PAP) (#19) for 18 mycotoxins ● Diet 1 contained low PP inclusion and diet 2 had high inclusion of PP.
Mosquitofish *(Gambusia affinis)*	AFB_1= 100^{1}; 215^{2}; 464^{3}; 1,000^{4}; 2,150^{5} µg L $^{-1}$ FB_1= 464^{6}; 1,000^{7}; 2,150^{8}; 4,640^{9}; 10,000^{10} µg L $^{-1}$ $^{11}AFB_1$:FB_1= 0:0 $^{12}AFB_1$:FB_1= 85.1/580 µg L $^{-1}$ $^{13}AFB_1$:FB_1=170.3/1,160 µg L $^{-1}$ $^{14}AFB_1$:FB_1=255.4/1,740 µg L $^{-1}$ $^{15}AFB_1$:FB_1=340.5/2,320 µg L $^{-1}$ $^{16}AFB_1$:FB_1=681.0/4,640 µg L $^{-1}$	McKean *et al.* 2006	n/a	n/a	n/a	n/a	↑17%^{2} ↑50%^{3} ↑100%^{4} ↑100%^{5} ↑17%^{8} ↑50%^{9} ↑67%^{10} ↑25%^{12} ↑25%^{13} ↑75%^{14} ↑91.7%^{15} ↑100%^{16}	n/a	● LC_{50} determinations in mosquitofish by probit analysis: AFB_1= 681 µg L^{-1}; FB_1= 4,640 µg L $^{-1}$ ● AFB_1:FB_1 correspond to 1/8; ¼; 3/8; ½ and 1.0

Nile tilapia (*Oreochromis niloticus*)	(AFB_1: 16[1] µg kg $^{-1}$ + OTA: 9[1] µg kg $^{-1}$)x Clay	Mohamed *et al*. 2010	n/a	n/a	↓PER[1] ↓SGR[1]	N	Y	n/a	● Initial weight: 1-3 g; 10 weeks study ● A mycotoxin binding material (Clay: calcium bentonite) was tested ● Represented parameters are compared to clay treatment (control)
Pacific white shrimp *(Litopenaeus vannamei)*	AFB_1= 0.3[1] and 10[2] µg ml $^{-1}$ FB_1= 1.4[3] and 2.0[4] µg ml $^{-1}$ AFB_1:FB_1 = 0.3:1.4[5]; 0.2:1.6[6]; 0.1:1.8[7] µg ml $^{-1}$	Perez-Acosta *et al.* 2016	↓HP ALP[1-4, W,C] ↓↓ HP ALP[5-7;W,C]	n/a	n/a	n/a	n/a	n/a	● Initial weight: 4.9 ± 2.8 g (wild shrimps)[W] ● Initial weight: 4.5 ± 1.7 g (captivity shrimps)[C] ● Clear synergistic effects were found when combining AFB_1 and FB_1 ● HP from the wild shrimp was less sensitive to the presence of the toxins evaluated.
Rainbow trout *(Oncorhynchus mykiss)*	GDDY*[1] inclusion of: (0, 15[1] and 30[2]%) x *MP (15% or 30%) *[1] Grain Distiller's Dried Yeast with OTA, DON, ZEN, FB_1 and FB_3	Hauptman *et al*. 2014	n/a	n/a	Y ↓WG[1,2] ↑FCR[2] ↑FI[2] ↓Fr[2] ↑VSI[2] ↑HSI[1,2]	n/a	n/a	n/a	● Initial weight: 26.4±0.9 g;12 weeks study ● Evaluated the effect of MTX deactivator (*[MP]Mycofix® Plus); (additional information about tested products at supplementary notes) ● Levels of MTX's in DDGS: 0.9 µg kg $^{-1}$ OTA; 7,000 µg kg $^{-1}$ DON; 133 µg kg $^{-1}$ ZEN; 2,000 µg kg $^{-1}$ FB_1; 1,000 µg kg $^{-1}$ FB_3; MTX's in experimental diets after inclusion of GDDY not analyzed.
Red Tilapia (*Oreochromis niloticus* × *O. mossambicus*)	*DON: 70[1], 310[2], 500[3], 920[4], 1150[5] + *ZEN: 10[1], 90[2], 210[3], 370[4], 980[5] µg kg $^{-1}$	Tola *et al*. 2015	L[2-5]	n/a	↓WG ↓TGC ↓FI ↓FE	AST, ALT, Hct, HSI	Y	n/a	● Initial weight: 7.5 g, 23 days study ● Diets *co-contaminated with other *Fusarium* metabolites: Aurofusarium [10-2,460 µg kg $^{-1}$]; Rubrofusarium [20-490 µg kg $^{-1}$]; Culmorin [20-1,390 µg kg $^{-1}$] and 15-Hydroxyculmorin [60-1,830 µg kg $^{-1}$]

Rainbow trout *(Oncorhynchus mykiss)*	[#1]FB_1= 0; 3,200[1]; 23,000[2] and 104,000[3] µg kg $^{-1}$ [#2]FB_1= (0; 3,200[4]; 23,000[5] and 104,000[6] µg kg $^{-1}$) x 24±1 ng g $^{-1}$ [^{3}H] AFB_1 [#3]AFB_1= (100 µg kg $^{-1}$) x FB_1= (0[7]; 3,200[8]; 23,000[9] and 104,000[10] µg kg $^{-1}$)	Carlson *et al.* 2001	[#1]No Tumors (L, K, S, GB) [#2]No Tumors (L, K, S, GB) [#3]↑↑ Tumors (K, S, GB) [#3]L[7]=35% [#3]L[8]=39% [#3]L[9]=61% [#3]L[10]=74%	n/a	n/a	[#1]↑Sa[2,3] [#1]↑So[2,3]	n/a	n/a	● Shasta strain rainbow trout; 60 weeks study ● [#1]FB_1 study: first fed phase of 4 weeks; recovery period of 6 weeks with posterior fed phase of 60 weeks. ● [#2] FB_1:AFB_1 synergism study: trout's fed 0; 3,200; 23,000 and 104,000 µg kg $^{-1}$ FB_1 and injected with 24±1 ng g $^{-1}$ [^{3}H] AFB_1 (0.84±0.04 µCi). ● [#3]AFB_1:FB_1 synergism study: trout's (0.5-1g) immersed in 100 µg kg $^{-1}$ AFB_1 for 30min followed by 4 weeks recovery and phase fed 0; 3,200; 23,000 and 104,000 µg kg $^{-1}$ FB_1 during 42 weeks
Rainbow trout *(Oncorhynchus mykiss)*	Control Control + HPDDG	Sealey *et al.* 2015	n/a	n/a	=WG, FCR, FI	n/a	n/a	n/a	● Initial weight: 30.5±1.6 g, ● Inclusion of HPDDG (high protein DDG) containing 2,500 µg kg $^{-1}$ DON and 1,643 µg kg $^{-1}$ ZEN; Mycotoxin levels in finished feed was not evaluated ● A anti-mycotoxin product (Biofix Plus) was supplemented (0.1%) to diets via vacuum-assisted top coating in the dietary oil portion. (additional information about tested products at supplementary notes)
Zebra fish *(Danio rerio)*	[IV, 1]50 µM CTN [IV, 2]200 µM PAT [IV, 3]15 µM CTN [IV, 4]50 µM CTN [IV, 5]200 µM PAT [IV,V, 6]15 µM CTN	Wu *et al.* 2012	[IV, 1,2]=K (Gt, Pt) [IV, 3-5]↑K(Hist)	↑COX2a[I V, V] ↑TNF[IV, V] ↑IL-1[V]	n/a	n/a	LC_{50} (µM) CTN/ PAT I= >100/ 239.9 II= 57.9/ 204.8 III= 28.3/ 183.7 IV= >100/ >250 V= 60.4/ >250 VI= 25.5/ >250		● Exposure life stages: I= 6-48hpf; II= 6-72hpf; III= 9-96hpf; IV= 24-48hpf; V= 24-72hpf; VI= 24-96hpf ● Exposure by bath ● After obtaining $LC_{50's}$, only IV and V were used to evaluate remaining effects
Zebrafish *(Danio rerio)*	[#1]DON= 0.0064, 0.064, 0.64, 6.4, and 64 µM [#2]ZEN= 0.0064, 0.064, 0.64, 6.4, and 64 µM [#3]PAT= 0.0064[1], 0.064[2], 0.64[3], 6.4[4], and 64[5] µM	Sidebotham 2015	[#1, #2]No toxicity	n/a	Hypo.aw[#3; 4,5] Learn.c[#3,]	n/a	Y[#3; 4,5]	n/a	● Tropical 5D wildtype adult zebrafish were used ● Exposition by bath; No co-exposition was tested ● Morphological and photomotor

	screenings were conducted at 24 hpf and 5 dpf.

Reference entries are in alphabetic order of species common name. Superscript letters give extra information; they are only valid for the same row.

Mycotoxins abbreviations: CTN= Citrinin; PAT= patulin

Physiological parameters abbreviations: ALP= Alkaline phosphatase; ALT= Alanine aminotransferase; AST= Aspartic aminotransferase; Ery=Erythrocyte count; FCR= Feed conversion ratio; FE= Feed efficiency; FI= Feed intake; Fr= Feeding rate; FW= Final weight; Hb=haemoglobin; Hct=haematocrit; Hep.N= hepatocyte nuclei; HSI= Hepatosomatic index; Hypo.aw= hypoactivity; IR= Inhibitory rate; LDH= lactate dehydrogenase; Learn.c= Learn capacity; Leuc= leucocytes; PCV= Packed-cell volume; PER= Protein efficiency ratio; Pyr= Serum pyruvate; RBCs= Total erythrocyte count (red blood cells); Sa/So= sphinganine/ sphingosine; Sa= sphinganin

SGR= Specific growth rate; So= sphingosine; TWG= Total weight gain; VSI= Visceral somatic index; WBCs= Total leucocyte count (white blood cells); WG= Weight gain.

Tissues or tissue related abbreviations: GB= gall bladder; Gt= glomeruli tubules; HP= Hepatopancreas; HSI= Hepatosomatic index; K= Kidney; L= Liver; Pt= pronephric tubules; S= Stomach.

Gene abbreviation: COX= cyclooxygenases; IL = interleukin; TNF= tumour necrosis factor

Table S1.13: Overall transfer of mycotoxins from feed to eggs, whole milk, meat and edible offal

Animal product	Transfer factors			Number of transfer factors used in calculation
	Mean±SD	Median	Maximum	
Eggs	0.18±0.49	0.007	5.5	66
Whole milk	0.10±0.18	0.0013	1.4	20
Meat	0.09±0.34	0.008	6	126
Edible offal	0.77±2.5	0.04	52	184
Fat	3.0±10	0.046	180	62

All data is taken from Leeman *et al.*, 2007.

Transfer factors are reported to edible offal (31%) and meat (25%), followed in about equal numbers by eggs (12%), whole milk (15%) and fat (17%). Animals included were cattle, poultry, pig, sheep, goat, rabbit, and several birds such as pheasant, turkey, duck and quail.

Transfer factors reported took into consideration the following mycotoxins: Aflatoxin B_1; Aflatoxin B_2; Aflatoxin G_1; Aflatoxin G_2; Deoxynivalenol; Ochratoxine A; T-2 toxin and Zearalenone.

Table S1.14: Documented aflatoxin carry-over on aquaculture species.

Reference	Species	Tested dosage	Mycotoxin detection level (µg kg^{-1})	Transfer factor	Method of analysis	Observations
Fish studies						
Suzy *et al.* 2017	African sharptooth catfish *(Clarias Gariepinus)*	10[1], 17[2] and 20[3] µg AFB_1 kg $^{-1}$	M[1] = 0.05±0.12 µg AFB_1 kg $^{-1}$ M[2] = 0.08±0.10 µg AFB_1 kg $^{-1}$ M[3] = 0.08±0.12 µg AFB_1 kg $^{-1}$	M[1] = 0.005 M[2] = 0.005 M[3] = 0.004	ELISA	▪ Initial weight: 4±2 g; 3 month study ▪ Chicken droppings were used as ingredient contaminated with 5, 7.2 and 8.2 µg AFB_1 kg $^{-1}$ ▪ Catfish fed 10 µg AFB_1 kg $^{-1}$ used as control ▪ No differences in haematological parameters
El-Sayed and Khalil, 2009	European seabass (Dicentrarchus labrax)	[#1]Oral 96h LC_{50} >0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 mg kg $^{-1}$ [#2]42 day exposure to 10% of oral 96h LC_{50} = 180 µg kg $^{-1}$	[#2]M = 4.25 ± 0.85 µg AFB_1 kg $^{-1}$	[#2]M = 0.236	ELISA	▪ Initial weight: 40±2 g ▪ [#1]96h LC_{50} = 0.18 mg kg $^{-1}$ bwt ▪ [#2]0.018 mg kg $^{-1}$ bwt AFB_1 ▪ [#1,2] Clinical signs: sluggish movement, loss of equilibrium, rapid opercular movement, and hemorrhages of the dorsal skin surface. [#2]Yellowish discoloration, pale discoloration of the gills, liver and kidney. Severe distension of the gall bladder.
Huang *et al.* 2011	Gibel carp *(Carassius gibelio)*	3.2, 11.3, 20.2[1], 55.2[2],95.8[3], 176.0[4] and 991.5[5] µg AFB_1 kg $^{-1}$	L[1-5] > 5 µg AFB_1 kg $^{-1}$ M[5] = 2.35 µg AFB_1 kg $^{-1}$	HP[1-5*]> 0.090 M[5] = 0.0024	ELISA	▪ Initial weight: 10.33±0.19 g ▪ 12 week study ▪ Fish showed strong clearance ability of AFB_1
Raghavan *et al.* 2011	Hybrid sturgeon *(Acipenser ruthenusx A. baeri)*	0, 1, 5, 10, 20, 40[1] and 80[2] µg AFB_1 kg $^{-1}$	M ≈ 28[1] and 34[2] L = 142.80[1] and 115.60[2] µg kg $^{-1}$	M[1] = 0.7 M[2] = 0.425 L[1] = 3.57 L[2] = 1.15	ELISA	▪ Initial weight: 10.53 ± 0.17 g ▪ 35 day study ▪ Liver hypertrophy and hyperchromasia of nuclei and cytoplasmic vacuoles, presence of inflammatory cells, focal hepatocyte necrosis and extensive biliary hyperplasia.
Lopes *et al.* 2005	Jundiá (*Rhamdia quelen*)	[#1] 41, 90[1] and 204[2] µg AFB_1 kg $^{-1}$ [#2] 350[1]; 757[2]; 1,177[3] µg AFB_1 kg $^{-1}$	[#1] M = 1[1] and 6.1[2] µg AFB_1 kg $^{-1}$ [#2] M+L=350[1]; 757[2] µg kg $^{-1}$ and 1,177[3] µg AFB_1 kg $^{-1}$	[#1] M[1] = 0.024 [#1] M[2] = 0.030 [#2]M+L[1] = 1 [#2]M+L[2] = 1 [#2]M+L[3] = 1	HPLC	▪ Initial weight: 3.21[#1] g and 4.73[#2] g ▪ 45[#1] and 35[#2] day studies
Michelin *et al.* 2016	Lambari fish *(Astyanax*	0, 10[1], 20[2] and 50[3] µg AFB_1 kg $^{-1}$	L = 265[2,t] and 243[3,t] µg kg $^{-1}$	L[2,t] = 13.25 L[3,t] = 4.86	HPLC	▪ Initial weight: 3.15 g ▪ 120 day study (sampling at day 30, 60, 90 and

	altiparanae)		M = 19[1,t], 20[2,t] and 50[3,t] µg kg $^{-1}$	$M^{1,t}$ = 1.9 $M^{2,t}$ = 1 $M^{3,t}$ = 1		120[t]) ▪ For the first 60 days of exposure, AFs were metabolised by liver and excreted. After 90 days, a lower efficiency in the elimination of AFs
Abdel Rahman *et al.* 2017	Nile tilapia *(Oreochromis niloticus)*	(0 and 200[1] µg AFB_1 kg $^{-1}$) x (FEO + SC)	L^1 = 5±0.5 µg AFB_1 kg $^{-1}$ M^1 = 3.7±0.1 µg AFB_1 kg $^{-1}$	L^1 = 0.025 M^1 = 0.019	HPLC	▪ Initial weight: 26.6±0.12 g; 30 day study ▪ Tested fennel essential oil (FEO) and saccharomyces cerevisiae (SC) as mycotoxin management strategy. ▪ AF effects are reported only for 0 and 200[1] µg kg $^{-1}$
Ayyat *et al.* 2013	Nile tilapia *(Oreochromis niloticus)*	(0, 250[1] µgAFB_1 kg feed $^{-1}$) x OZ, B or C	M^1 = 78.33 µg kg $^{-1}$	M^1 = 0.313	HPLC	▪ Initial weight: 7.3 g; 3 week study ▪ Tested ozone (0.5 mg/L/minute; OZ), bentonite (20 g kg $^{-1}$ diet; B) and coumarin (5 g kg $^{-1}$ diet; C) as detoxifying strategy
Deng *et al.* 2010	Nile tilapia *(Oreochromis niloticus)*	19; 85[0]; 245[1]; 638[2]; 793[3] and 1,641[4] µg kg $^{-1}$	$Y^{t1\text{-}tf;0\text{-}4}$ L^{t1} = 10[0], 16[1], 21[2], 24[3] and 24[4] µg AFB_1 kg $^{-1}$ liver L^{tf} = 30[0], 33[1], 47[2], 44[3] and 43[4] µg AFB_1 kg $^{-1}$ liver	$Y^{t1\text{-}tf;0\text{-}4}$ L^{t1} = 0.118[0], 0.065[1], 0.033[2], 0.030[3] and 0.015[4] L^{tf} = 0.353[0], 0.135[1], 0.074[2], 0.055[3] and 0.026[4]	ELISA	▪ Initial weight: 20 g; ▪ 20[tf] week study (sampling at week 5[t1]) ▪ AF from mouldy peanut meal
Hessein *et al.* 2014	Nile tilapia *(Oreochromis niloticus)*	(0, 250[1] mg kg $^{-1}$) x Vit or C	M^1 = mg kg $^{-1}$	M^1 = 0.407	HPLC	▪ Initial weight: 7.3 g; 98 day study ▪ Tested coumarin (5 g kg $^{-1}$ diet; C) and vitamin E (50mg kg $^{-1}$ diet; Vit) as detoxifying strategy ▪ No differences on Hb, RBcs, Hct, WBCs, Plat Note: Hessein *et al.*, 2014 reports in his manuscript a residual AF of 107.7 mg kg $^{-1}$, each seems extremely high, which might be a mistake of units mg kg $^{-1}$/ µg kg $^{-1}$
Hussain *et al.* 2017	Nile tilapia (*Oreochromis niloticus*)	(0, 2000[1], 4000[2] mg kg $^{-1}$) x 0.5% and 1% CB	M^2 = 0.087±1.32 µg kg $^{-1}$	M^2 ~ 0	HPLC	▪ Initial weight: 4.5±0.4 g; 10 week study ▪ Tested calcium bentonite (CB) clay as detoxifying strategy; ▪ Tested CB significantly improved some parameters (WG, HIS) ▪ CB significantly reduced bioaccumulation of AFB_1 residues in muscle tissues.
Mahfouz *et*	Nile tilapia	20[1] and 100[2] µg AFB_1 kg $^{-}$	L	$L^{1,t1}$ = 0.25	TLC	▪ Initial weight: 35±0.50 g; 6[t1] or 12[t2] week

***al*. 2015**	*(Oreochromis niloticus)*	[1] feed	[1,t1] = 5 µg kg $^{-1}$ [1,t2] = 8 µg kg $^{-1}$ [2,t1] = 10 µg kg $^{-1}$ [2,t2] = 15 µg kg $^{-1}$ M[2; t2] = 5 µg kg $^{-1}$	L [1,t2] = 0.4 L [2,t1] = 0.1 L [2,t2] = 0.15 M[2; t2] = 0.05		studies ▪ Challenge test with *Aeromonas hydrophila, IP* ▪ Expression of liver *GPx* and *GST* down-regulated[1] ▪ The ability to withstand *A. hydrophila* infection was remarkably lowered
Salem *et al*. 2009	Nile tilapia *(Oreochromis niloticus)*	0, 150[1] µg AFB_1 kg $^{-1}$	M[1] = 99.48 µg AFB_1 kg $^{-1}$	M[1] = 0.663	HPLC	▪ Initial weight: 10±3 g; 15 week study ▪ AFB_1 was produced through pellet fermentation using *Aspergillus parasiticus* NRRL 2999
Selim *et al*. 2014	Nile tilapia *(Oreochromis niloticus)*	(0 and 200[1] µg kg $^{-1}$) x HSCAS, SC and EGM	M[1] ≈ 90 µg kg $^{-1}$	M[1] ≈ 0.45	HPLC	▪ Initial weight: 15±2 g; 10 week study ▪ Tested hydrated sodium calcium aluminosilicates (HSCAS; 0.5%), *Saccharomyces cerevisiae* (S.C.; 0.25%) and an esterified glucomannan (EGM; 0.25%) as detoxifying strategy; ▪ AF produced from polished raw rice
Ngethe *et al*. 1993	Rainbow trout *(Oncorhynchus mykiss)*	15.6 µg ml $^{-1}$ of AFB_1	L[1, 2, 4] B[1, 2, 4]	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	▪ Initial weight: 200±20 g; 3 week study (sampling at 6h[1], 1 day[2], 2 days[3] and 6 days[4]) ▪ Intravenous injection of 3H-AFB_1
Ellis *et al*. 2000	Rainbow trout *(Oncorhynchus mykiss)*	20 µg kg $^{-1}$ AFB1 and 20 µg kg $^{-1}$ AFB_1 + 2% clay	Detected in: F, K, GI, U, Bi, Ca	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	▪ Initial weight: 266±12.6 g, 7 day study ▪ 2% sodium bentonite Volclay tested as detoxifying strategy;
Ngethe *et al*. 1992;	Rainbow trout *(Oncorhynchus mykiss)*	15.6 µg ml $^{-1}$ of AFB_1	Detected in: Bi, L, K, B, AbF, M, Sp and Bl	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	▪ Initial weight: 100±15 g, 8 day study (sampling at 6h, 1, 2 4 and 8 days) ▪ Intravenous injection and oral dose of ^{3}H-AFB_1
Usanno *et al*. 2005	Red tilapia *(Oreochromis niloticus x O. mossambicus)*	0, 50, 100, 500, 1,000 and 2,500 µg kg $^{-1}$	Not detected	n/a	n/a	▪ 8 week trial ▪ No information on fish weight

Husssain *et al.* 1993	Walleye fish *(Sander vitreus)*	0, 50 and 100[1] µg kg $^{-1}$	Detected in muscle: AFB_1[1] = 5 µg kg $^{-1}$ AFB_2[1] = 10 µg kg $^{-1}$ AFG_1[1] = 15 µg kg $^{-1}$ AFG_2[1] = 20 µg kg $^{-1}$	AFB_1 = 0.5 AFB_2 = 0.1 AFG_1 = 0.15 AFG_2 = 0.2	n/a	▪ 30 day study ▪ No information on fish weight
Shrimp studies						
Boonyaratpalin *et al.* 2001	Black tiger shrimp *(Penaeus monodon Fabricius)*	0; 50[1]; 100[2]; 500[3]; 1,000[4]; 2,500[5] µg kg $^{-1}$ AFB_1	Head and shell / muscle (µg kg $^{-1}$) [1,t1] = 2.6/13.0; [1,t2] = 0.5/ 0.4 [2,t1] = 3.5/ 14.2; [2,t2] = -/ 0.6 [3,t1] = 9.1/ 10.6; [3,t2] = 6.8/ 0.3 [4,t1] = 2.3/8.4; [4,t2] = 6.5/0.7 [5,t1] = 3.9/7.4; [5,t2] = 4.9/0.1	Head and shell / muscle (µg kg $^{-1}$) [1,t1] = 0.052/0.26; [1,t2] = 0.01/ 0.008 [2,t1] = 0.035/ 0.142; [2,t2] = -/ 0.006 [3,t1] = 0.0182/ 0.0212; [3,t2] = 0.0136/ 0.0006 [4,t1] = 0.0023/0.0084; [4,t2] = 0.0065/0.0007 [5,t1] = 0.0016/0.0030; [5,t2] = 0.0020/~0	TLC	▪ Study in adult stage, Initial weight: 1.0-1.2 g; 8 week trial (sampling at 4[t1] and 6[t2] weeks)
Bintvihok *et al.* 2003	Black tiger shrimp (Penaeus monodon Fabricius)	5, 10, 20 µg kg $^{-1}$ AFB_1	not detected	n/a	HPLC	▪ Study in adult stage ▪ 10 day trial ▪ AFB_1 was prepared from mouldy corn
Bautista *et al.* 1994	Black tiger shrimp (Penaeus monodon Fabricius)	25, 50, 75, 100 or 200 µg kg $^{-1}$ AFB_1	not detected	n/a	HPTLC	▪ Study in adult stage, Initial weight: 17.5±0.6 g ▪ 62 day trial

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding the mycotoxin contamination, when not mentioned it is assumed that a purified form of the respective mycotoxin was used.
General abbreviations: HPLC = High-performance liquid chromatography; ELISA = enzyme linked immunosorbent assay; TLC = Thin layer chromatography; LOD = limit of detection; nd = not detected; n/a = not applicable.
Tissue abbreviations: M = Muscle; L = Liver; HP = hepatopancreas; B = Brain; F = faeces; K = Kidney; GI = Gastro intestinal tract; U = Urine; Bi = Bile; Ca = carcass; AbF = abdominal fat; Sp = spleen and Bl = blood.

Table S1.15: Documented ochratoxin carry-over in aquaculture species.

Reference	Species	Tested dosage	Mycotoxin detection level (µg kg $^{-1}$)	Transfer factor	Method of analysis	Observations
Fish studies						
Bernhoft *et al.* 2017	**Atlantic Salmon** (*Salmo salar*)	0, 800[1] and 2400[2] µg kg $^{-1}$ OTA	L/M/K/SK (µg kg $^{-1}$) [1,t1] = 1.86/<LOQ/n.s./n.s. [1,t2] = 1.53/<LOQ/n.s./n.s. [1,t3] = 1.01/<LOQ/0.16/n.s. [2,t1] = 4.81/ <LOQ/n.s./n.s. [2,t2] = 3.27/ <LOQ /n.s./n.s. [2,t3] = 2.61/ <LOQ/1.03/n.s.	L/M/K/SK [1,t1] = 0.0023/<LOQ/n.s./n.s. [1,t2] = 0.0020/<LOQ/n.s./n.s. [1,t3] = 0.0012/<LOQ/~0/n.s. [2,t1] = 0.0020/<LOQ/n.s./n.s. [2,t2] = 0.0013/<LOQ /n.s./n.s. [2,t3] = 0.0011/ <LOQ/~0/n.s.	HPLC	▪ Initial weight: 58 g ▪ Administration of ^{14}C-OTA A and autoradiography ▪ Sampling at 3[t1], 6[t2] and 8[t3] weeks
Fuchs *et al.* 1986	**Rainbow trout** (*Salmo gairdneri*)	IV injection of 0.160 µg kg $^{-1}$	Blood = Detected [t1-t4] Pronephros = Detected [t1-t4] Opisthonephros = Detected [t1-t4] Urine = Detected [t1-t4] Pseudobranch = Detected [t1-t4] Gills = Detected [t1-t4] Liver = Detected [t1-t4] Bile = Detected [t1-t4] Ventricle wall = Detected [t1-t4] l'yloric appendices = (contents) = Detected [t1-t4] Large intestine (contents) = Detected [t1-t4] Splccn ("patches") = Detected [t1-t4] Muscle (close to the myomeres) = Detected [t1-t2] Spinal cord = Detected [t1-t3] Fins = Detected [t1-t4] Skin = Detected [t1-t4] Muscles = Detected [t1-t2]	n/a	LC fluorometer	▪ Initial weight: 50 g, 8 week study ▪ Sampling at 5 min[t1], 6[t2] and 8[t3] weeks ▪ Fish each was sacrificed at 5[t1] min, 1[t2] hr, 24[t3] hrs and 8[t4] days after injection.
Shrimp studies						
Supamattaya *et al.* 2005	**Black tiger shrimp black** (*Penaeus monodon Fabricius)*	100; 200 and 1,000 µg kg $^{-1}$	Not detected	n/a	HPLC	▪ Initial weight: 2 g; 8 week study ▪ No differences on THC or Ca^{2+} levels ▪ No differences in tissues: G, AG, HP, HT, * LOD given in the manuscript (44,000 µg kg $^{-1}$) seems to be very high; there is a chance of an error in the units

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC = High-performance liquid chromatography; LC = liquid chromatography; n/a = not applicable; n.s. not sampled

Tissue abbreviations: M = Muscle; L = Liver; K = Kidney; SK = skin.

Table S1.16: Documented zearalenone and T-2 carry-over in aquaculture species.

Reference	Species	Tested dosage	Mycotoxin detection level (µg kg $^{-1}$)	Transfer factor	Method of analysis	Observations
Fish studies						
Pietsch *et al.* 2015	**Common Carp** (*Cyprinus carpio* L.)	0; 332[1]; 621[2] and 797[3] µg kg $^{-1}$	Muscle ZEN[1] = 0.13±0.03 µg kg $^{-1}$ ZEN[2] = 0.22±0.18 µg kg $^{-1}$ ZEN[3] = 0.15± 0.07 µg kg $^{-1}$ α-ZEN[1] = 0.11±0.03 µg kg $^{-1}$ α-ZEN[2] = 0.16±0.011 µg kg $^{-1}$ α-ZEN[3] = 0.05± 0.07 µg kg $^{-1}$ ZEN[1, RP] = 0.03±0.03 µg kg $^{-1}$ ZEN[2, RP] = 0.03±0.02 µg kg $^{-1}$ ZEN[3, RP] = 0.03±0.03 µg kg $^{-1}$	Muscle ZEN[1] ~ 0 ZEN[2] ~ 0 ZEN[3] ~ 0 α-ZEN[1] ~ 0 α-ZEN[2] ~ 0 α-ZEN[3] ~ 0 ZEN[1, RP] ~ 0 ZEN[2, RP] ~ 0 ZEN[3, RP] ~ 0	HPLC	● Raised from egg with 12-16 cm in length ● 4 week study ● α-ZEN were not detectable after recovery period (2 weeks) and ZEN was detected at 0.03 µg kg $^{-1}$ dry weight for all treatments
Woźny *et al.* 2015	**Rainbow trout** (*Oncorhynchus mykiss*)	1,810 µg kg $^{-1}$	Intestines ZEN = 732.2 µg kg $^{-1}$ α-ZEN = 10.7 µg kg $^{-1}$ L = residual ZEN and α-ZEN in all sampled fish	Intestines ZEN = 0.40 α-ZEN = 0.0059	HPLC	● Initial weight: 250 g, all females; 71 day study ● Some animals were identified as males ● ZEN was detected (<5.0 µg kg $^{-1}$) in all female ovaries
Woźny *et al.* 2017	**Rainbow trout** (*Oncorhynchus mykiss*)	1 mg kg $^{-1}$ of body mass	ZEN/α-ZEN/β-ZEN (µg kg $^{-1}$) I[48h] = ~1500/~600/- I[96h] = ~1500/~900/- L[48h] = ~700/~100/~500 L[96h] = <200/<20/~0 O[48h] = 321/~100/- O[96h] = <100/<100/- Oo[48h] = ~25/~10/- Oo[96h] = <5/<5/- P[48h] = ~10/~5/- P[96h] = ~0/~0/- M[48h] = ~5/~5/- M[96h] = ~3/~3/-	ZEN/α-ZEN/β-ZEN (µg kg $^{-1}$) I[48h] = 1.5/ 0.6/- I[96h] = 1.5/ 0.9/- L[48h] = 0.7/ 0.1/ 0.5 L[96h] = <0.2/<0.02/~0 O[48h] = 0.321/ 0.1/- O[96h] = <0.1/<0.1/- Oo[48h] = ~0.025/~0.01/- Oo[96h] = <0.005/<0.005/- P[48h] = ~0.01/~0.005/- P[96h] = ~0/~0/- M[48h] = ~0.005/~0.005/- M[96h] = ~0.003/~0.003/-	HPLC-FLD	● Initial weight: 1274±162 g, all mature females ● Objective was to study the ZEN carry-over to eggs ● Administration on ZEN – oral (bolus) ● Sampling periods: 2, 6, 12, 24, 48, 72, 96h ● Verified the presence of ZEN and α-ZEN in commercial fish roe ● "Contamination of fish roe with zearalenone residuals is unlikely to pose a health risk to consumers, but their potential to transfer to somatic cells in fish ovaries may be of concern for aquaculture", Woźny *et al.* 2017
Shrimp - no studies for ZEN; Shrimp – T-2						
Deng et al. 2017	**Pacific white shrimp** (*Litopenaeus vannamei)*	0; 500[1]; 1,200[2]; 2,400[3]; 4,800[4]; 12,200[5] µg kg $^{-1}$ T-2	[HP]m= 17.52±2.87[4] ηg g $^{-1}$ [HP]m= 48.61±3.13[5] ηg g $^{-1}$	n/a	TSQ	● Initial weight: 8.5±0.5 g; 20 days study ● Dietary concentrations correspond to $^{1}/_{50}$, $^{1}/_{20}$, $^{1}/_{10}$, $^{1}/_{5}$ and $^{1}/_{2}$ (Wang *et al.* 2015).

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC = High-performance liquid chromatography; HPLC-FLD = High-performance liquid chromatography with fluorescence detection; TSQ= Quantum Access tandem mass spectrometer

Tissue abbreviations: I = Intestines; O = Ovaries; Oo = Oocytes; P = Plasma, M = Muscle

Table S1.17: Regulatory limits and guidance values for mycotoxins in animal feed in the EU applicable to aquaculture feedstuffs. Iceland, Norway and Liechtenstein are following EU legislation for Aflatoxins and Ergot alkaloids. Limits in µg kg^{-1} (ppb) or mg kg^{-1}(ppm) relative to a feeding stuff with a moisture content of 12%.

	Maximum levels
Aflatoxins	
Commodity	**Limit (ppb)**
All feed materials	20
Complementary and complete feed	10
	Guidance values
Fumonisins (B_1 + B_2)	
Commodity	**Limit (ppb)**
Maize and maize products	60000
Complementary and complete feeding stuffs for fish	10000
Ochratoxin A	
Commodity	**Limit (ppb)**
Cereals and cereal products	250
Deoxynivalenol	
Commodity	**Limit (ppb)**
Cereals and cereal products with the exception of maize by-products	8000
Maize by-products	12000
Complementary and complete feeding stuffs	5000
Zearalenone	
Commodity	**Limit (ppb)**
Cereals and cereal products with the exception of maize by-products	2000
Maize by-products	3000

References: Commission Regulation (EC) No 1881/2006; Commission Regulation (EC) No 1137/2015; Commission Regulation (EC) No 1126/2007; Commission Regulation (EC) No 239/2016; Commission Regulation (EC) No 105/2010; Directive 2002/32/EC; Commission Regulation (EC) No 165/2010; Commission Recommendation 2006/576/EU; Commission Recommendation 2013/165/EU; Commission Recommendation 2013/627/EU; Commission Regulation (EC) No 212/2014; Commission Recommendation 2016/1319/EU.

Table S1.18: Regulatory limits and guidance values for mycotoxins in animal feed in the EU. Iceland, Norway and Liechtenstein are following EU legislation for Aflatoxins and Ergot alkaloids. Limits in µg kg $^{-1}$ (ppb) or mg kg $^{-1}$ (ppm) relative to a feeding stuff with a moisture content of 12%.

Maximum levels	
Aflatoxins	
Commodity	**Limit (ppb)**
All feed materials	20
Complementary and complete feed	10
With the exception of:	
Compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	5
Compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals)	20
Ergot alkaloids / Rye Ergot (*Claviceps purpurea*)	**Limit (ppm)**
Feed materials and compound feed containing unground cereals	1000
Guidance values	
Fumonisins (B_1 + B_2)	
Commodity	**Limit (ppb)**
Maize and maize products	60000
Complementary and complete feedingstuffs for	
pigs, horses (*Equidae*), rabbits and pet animals	5000
fish	10000
poultry, calves (< 4 months), lambs and kids	20000
adult ruminants (> 4 months) and mink	50000
Ochratoxin A	
Commodity	**Limit (ppb)**
Cereals and cereal products	250
Complementary and complete feed stuffs for:	
Cats and dogs	10
pigs	50
poultry	100
Deoxynivalenol	
Commodity	**Limit (ppb)**
Cereals and cereal products with the exception of maize by-products	8000
Maize by-products	12000
Complementary and complete feeding stuffs	5000
With the exception of:	
Complementary and complete feeding stuffs for pigs	900
Complementary and complete feeding stuffs for calves (< 4 months), lambs, kids and dogs	2000
Zearalenone	
Commodity	**Limit (ppb)**
Cereals and cereal products with the exception of maize by-products	2000
Maize by-products	3000
Complementary and complete feeding stuffs for:	
piglets and gilts (young sows)	100
sows and fattening pigs	250
calves, dairy cattle, sheep (including lamb) and goats (including kids)	500

Table S3.1: Analysed mycotoxin/ metabolites content of the basal experimental diet. Values in µg kg $^{-1}$

Analyte	Value Found	Analyte	Value Found
Major Mycotoxins		***Fusarium* metabolites**	
Aflatoxin B_1	Not detected	15-Hydroxyculmorin	117.30
Zearalenone	78.63	Culmorin	205.16
Deoxynivalenol	37.42	Aurofusarin	224.30
T-2 Toxin	Not detected	Moniliformin	114.30
Fumonisin B_1	67.73	***Penicillium* Toxins**	
Ochratoxin A	2.00	Brevianamid F	1,409.39
Sum of Ergot alkaloids	Not detected	Rugulusovin	541.11
Alternaria Toxins		***Aspergillus* Toxins**	
Alternariol	0.42	Tryptophol	146.70
Zearalenone-Derivates		**Other metabolites**	
Zearalenone-sulfate	detected	Cyclo(L-Pro-L-Val)	3,774.00
		Cyclo(L-Pro-L-Tyr)	5,398.57

Table S3.2: Forward and reverse primers, accession numbers, amplicon size, amplification efficiency and error for each pair of primers used for the experimental work.

Tissue	Genes	Forward (5'-3')	Reverse (5'-3')	Accession numbers	Amplicon size (bp)	E (%)	R^2
Head Kidney	*ef1a*	ATCGGCGGTATTGGAACAGT	TGGTGCATCTCCACAGACTT	NM_001124339.1	122	92.01	0.999
	actb	CGTCCACCGTAAATGCTTCT	CCCTCTCTCTCTCCCTCTGG	NM_001124235.1	118	92.56	0.999
	star	CATCAAAGGCACTCCAAGAGA	GAAGGAAAGGACATGAGGAGA	NM_001124202.1	150	106.87	0.997
Liver	*ef1a*	ATCGGCGGTATTGGAACAGT	TGGTGCATCTCCACAGACTT	NM_001124339.1	122	99.61	0.999
	actb	CGTCCACCGTAAATGCTTCT	CCCTCTCTCTCTCCCTCTGG	NM_001124235.1	118	101.47	1.000
	Igf1	GCAGTTTGTGTGTGGAGAGAG	TCTGGAAGCAGCACTCGTC	NM_001124696.1	110	103.78	0.999
	Igf2	AAATATGAGGCGTGGCAGAG	TCTTGGGCCTTGATCTTCAC	NM_001124697.1	110	100.11	0.998
Brain	*ef1a*	ATCGGCGGTATTGGAACAGT	TGGTGCATCTCCACAGACTT	NM_001124339.1	122	95.99	0.996
	actb	CGTCCACCGTAAATGCTTCT	CCCTCTCTCTCTCCCTCTGG	NM_001124235.1	118	102.44	0.999
	crf1	CAAGGCAAAGTTGGGAACAT	ATCATCTGTCGGAGCATGTG	NM_001124286.1	137	97.16	0.998
	crf2	ATAGGTTCATCAGCGGCTTC	CTCCATCATCTGTCGAAGCA	NM_001124627.1	119	100.91	0.997
	crfbp	GTGGTGAGGATGGTGTCCA	CAGCCAGTGAAGCAGAAATC	NM_001124631.1	125	102.74	0.988
	npy	CAAGGCAGAGGTATGGGAAG	CCACAACGAGGGTTCATCAT	NM_001124266.1	122	98.08	0.999
	adcyap1a	GGCAAAGCGTGTAAGTGGAG	CCGCCAGGTATTTCTTGACT	NM_001124297.1	134	99.80	0.998
	lep	TGCGCTAAACAGACTCAAGG	CAGCATGGCACAAACTGATC	NM_001145890.1	122	98.63	0.995
GIT	*ef1a*	ATCGGCGGTATTGGAACAGT	TGGTGCATCTCCACAGACTT	NM_001124339.1	122	99.61	1.000
	actb	CGTCCACCGTAAATGCTTCT	CCCTCTCTCTCTCCCTCTGG	NM_001124235.1	118	100.61	0.996
	sst2	CCTGCTCCATACCGACTGAT	CAGACCTTCATCTCCAACAGA	NM_001124703.1	111	94.78	0.999
	chia	ACGCCTACACCCAGCAGA	AAGGTTCCAGAGAAGTCATCCA	NM_001160474.1	139	94.65	0.998
	pga	GGTGGGTGTCAGGCTATCAT	ACGGTGGCATCTCCATACTG	NM_001160475.1	119	98.19	0.996
	lpl	GGTGCATTTCTTCAGCAGTG	CAGGAAGGGCATGGTGTAAG	NM_001124604.1	112	98.57	0.994
	ghrl	TGATGCTGTGTACTCTGGCTCT	GGCTTCCCTTTACCCTGTCT	NM_001124588.1	103	97.44	0.999
	cel1	CACTCGCTAACCTCCCTCTG	TGGTCTCCTGACTGGGCTTA	FR904683.1	132	93.07	0.990
	cel2	CACGCACCTACTCCTACCTCTT	GCTTGCCGAACACATACTGC	FR904649.1	117	96.97	0.988
	cckt	TCCAACACTCTCTCTCTGTCTCC	GTGTGCGTCCTGAACAGCTA	NM_001124344.1	105	99.54	0.996
	cckn	AACACTCCTCCAACACAAGGA	TCCCAAACAGCTAGTGGACA	NM_001124611.1	96	99.63	0.999
	cckl	CACACCTCTCCTCTACTGTTATCA	GAGAACGCAGCCAGCAGT	NM_001124345.1	105	97.02	0.999
	amy2a	CGTGAAATGCAAACAACAGC	TGAAGAGTGCAGAAGAGTGCA	FR904634.1	100	103.96	0.987

	atp4a	AAAGACACACCTCCATGTTCTAA	AAAGGCAATCACACACAGACA	FR906150.1	129	104.32	0.998
	crtl	GTTGTCACTGCTGCCCATTG	TGATAGCCCTGGAGATGCTT	FR904735.1	118	100.67	0.999
	try1	CAGGATCATACAGCAACCATG	CTTGCACTCATACCCTCCAA	*	102	96.82	1.000
	try2	AATGGCAGAGGACAGAGTGG	GCGAACAGAGCGAGAAGAAT	FR904735.1	107	100.78	0.982
	try3	GTGGAGGAGAGGGATGAGAA	CACCGCAGAAGTGGTAGCC	FR904447.1	100	96.18	0.999

Note: * Conserved between deduced sequences from FR908792.1, FR909975.1 and FR912004.1 (several isoforms).

Scientific publications, presentations and awards during PhD course

Scientific publications in peer-reviewed journals

- **CHAPTER 1.3 - Consequences of mycotoxins for aquaculture (Literature review) was based on:**

Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: a real risk to aquaculture?

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And

Mycotoxins in Aquaculture: Feed and Food

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Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: a real risk to aquaculture?

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Abstract

There is increasing awareness of the negative effects of mycotoxins in aquatic species, which is highlighted in recent publications. Partly due to climate change associated with an overall increase of mycotoxins contamination in plant ingredients, and also due to the tendency to replace expensive animal-derived proteins, such as fish meal, by more economical plant proteins sources, which increases the probability of mycotoxin contamination in aquaculture feeds (Hooft *et al.* 2011). Over a 1-year period, 41 samples of finished aquaculture feed, both shrimp and fish, were analysed within the scope of BIOMIN mycotoxin survey programme. The samples were tested for aflatoxins, zearalenone, deoxynivalenol, fumonisins and ochratoxin A. Samples were sourced in Asia (31 samples) and Europe (10 samples) from fish/shrimp farms or feed producers. The values detected pose a risk for several important aquaculture species, assuming single mycotoxin contamination, that is excluding possible additive and synergetic effects between mycotoxins. Co-occurrence of mycotoxins in feeds may induce synergistic effects and increase the negative impact of mycotoxins in aquatic-farmed species at lower levels than when present in single contamination. This review gives an overview of the different mycotoxins and revises the effects of mycotoxins in aquatic species. Additionally, it reports the levels of mycotoxins in aquafeeds in 2014 and compares detected levels with possible negative effects in fish and shrimp. As it is highlighted by the results of the survey, the risk of co-occurrence is high and the knowledge on the effects of multimycotoxins contamination in aquatic species is basically none.

Key words: carry-over effects, co-occurrence, fish, moulds, shrimp.

Introduction

Mycotoxins are toxic secondary metabolites produced by moulds (Hussein & Brasel 2001). They can be produced on agricultural commodities before and/or after harvest; during transportation or storage, they can cause adverse health outcomes when consumed by humans and animals. Some mycotoxicoses, the toxic manifestations of mycotoxins in humans or animals, have been known for hundreds of years, for example ergotism. It is thought that mycotoxins have plagued mankind since the beginning of controlled crop production (FAO 2001) if not earlier. Chemically, mycotoxins display a wide range of structures, differing also in biological effects, for example carcinogenic, teratogenic, mutagenic, oestrogenic, neurotoxic or immunotoxic (Abd-Allah *et al.* 1999; McKean *et al.* 2006c; El-Sayed & Khalil 2009; El-Sayed *et al.* 2009; Hooft *et al.* 2011). They account for millions of American dollars lost annually worldwide due to negative impacts on human health, animal health and condemned agricultural products (Shane 1994; Vasanthi & Bhat 1998; Hussein & Brasel 2001; CAST 2003). Mycotoxins are chemically and thermally stable, rendering them unsusceptible to commonly used feed manufacturing techniques such as extrusion (Kabak *et al.* 2006; Leung *et al.* 2006).

The importance of mycotoxins to livestock production and aquaculture first became apparent during the early 1960s with outbreaks of aflatoxicosis in young turkeys in the United Kingdom and hatchery-reared rainbow trout (*Onchorhynchus mykiss*) in the United States. In both cases, the origin of aflatoxicosis was aflatoxin-contaminated feed (peanut meal for turkeys and cottonseed meal for trout)

(Wolf & Jackson 1963; Kumar *et al.* 2013). The wide concern about mycotoxins, last years, has been growing due to the climate change, which has been associated with the overall increase of mycotoxins contamination in ingredients of plant origin. It has been documented that, for example, changes in temperature and precipitation patterns can affect infection of crops by toxigenic fungi, the growth of these fungi and the production of mycotoxins (Tirado *et al.* 2010). In aquaculture, the tendency and the economical need to replace expensive animal-derived proteins, such as fish meal, with less expensive plant proteins sources, have also increased the impact of mycotoxin contamination in aquaculture feeds (Hooft *et al.* 2011).

Mycotoxins in aquafeed

Research characterizing the adverse effects of mycotoxins on the performance and health of animals has in large part focused on terrestrial livestock species (Rotter *et al.* 1996; D'Mello & Macdonald 1997; Pestka 2007). However, since the 1960s with outbreak of aflatoxicosis in trout, research has been carried out on the effects of mycotoxins in aquaculture species. This became even more important with the high cost of fish meal and the need to identify more economical protein sources, such as plant protein or other commercially available by-products (e.g. dried distillers grains and soluble; DDGS). In the case of DDGS, the final by-product may contain higher concentrations of mycotoxins relative to the grains from which they are derived (Schaafsma *et al.* 2009). Generally, most of the mycotoxins that have the potential to reduce growth and health status of aquaculture-farmed animals are produced by *Aspergillus*, *Penicillium* and *Fusarium* sp. Toxic metabolites produced by these fungi are known to be either carcinogenic (e.g. aflatoxin B1, ochratoxin A, fumonisin B1), oestrogenic (zearalenone), neurotoxic (fumonisin B1), nephrotoxic (ochratoxin), dermatotoxic (trichothecenes) or immunosuppressive (aflatoxin B1, ochratoxin A and T-2 toxin).

Aflatoxins

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *A. parasiticus*. *Aspergillus flavus* is particularly common contaminant in agriculture. *Aspergillus bombycis*, *A. ochraceoroseus*, *A. nomius* and *A. pseudotamari* are also aflatoxin-producing species, but they are encountered less frequently (Hussein & Brasel 2001). They were the identified cause of the Turkey X disease (i.e. hepatic necrosis) in 1960 (Asao *et al.* 1963). The main aflatoxins commonly found in aquaculture feedstuffs are aflatoxin B1, (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) (Ottinger & Kaattari 1998, 2000; Huang *et al.* 2011). These mycotoxins occur especially in subtropical and tropical areas contaminating mainly feedstuffs with high starch and lipid content, such as cottonseed, corn, peanut, wheat and soya bean (Ostrowski-Meissner *et al.* 1995). AFB1 is one of the most potent carcinogens known, classified as a group I carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer (IARC) 1993) and highly hepatocarcinogenic (Busby & Wogan 1984; Sharma & Salunkhe 1991; Miller & Trenholm 1994; Wang *et al.* 1998, 2008). The biological effects of AFB1 in these aquatic species are thought to be directly linked to the toxin's concentration in feed and to animal's age and species (Hendricks 1994).

Zearalenone

Zearalenone (ZEN) is a phytoestrogenic compound (Diekman & Green 1992) known as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid μ-lactone. It is a metabolite primarily associated with several *Fusarium* species (i.e. *F. culmorum*, *F. graminearum*, and *F. sporotrichioides*). *Fusarium graminearum* is the fungus mainly responsible for the oestrogenic effects commonly found in farm animals (Marasas 1991). These species are regular contaminants of cereal crops worldwide (Hussein & Brasel 2001). ZEN resembles 17β-estradiol, the principal hormone produced by the vertebrate's ovaries. As a hormone mimicking substance, ZEN can bind to oestrogen receptors in target cells (Kumar *et al.* 2013). ZEN studies have focused mainly on dysfunction or structural disorders in the reproductive tract of farm animals (Zinedine *et al.* 2007; Minervini & Aquila 2008; Woźny *et al.* 2013). It has been shown that the oestrogenic properties of ZEN give rise to a number of reproductive disorders in exposed livestock mammals, including decreased libido, anovulation, infertility or neoplasmic lesions (Zinedine *et al.* 2007; Minervini & Aquila 2008). Similarly in oviparous animals, including fish, ZEN mimics the action of natural oestrogen, 17β-estradiol, by binding to cells' oestrogen receptors (ERs) and activating responsive genes that encode vitellogenin or zona radiata protein – major structural elements of the oocyte (Arukwe *et al.* 1999; Woźny *et al.* 2008; Chen *et al.* 2010).

Trichothecenes

Trichothecenes are compounds containing sesquiterpene rings characterized by a 12, 13-epoxy-trichothec-9-ene nucleus. They are mainly produced by several *Fusarium* species (e.g. *F. sporotrichioides*, *F. graminearum*, *F. poae* and *F. culmorum*), but can also be produced by members of other genera such as *Myrothecium* (Tamm & Breitenstein 1984) and *Trichothecium* (Jones & Lowe 1960). Trichothecenes include T-2 toxin, diacetoxyscirpenol (DAS),

deoxynivalenol (known as DON or vomitoxin) and nivalenol. Both T-2 toxin and DAS are the most toxic and are soluble in non-polar solvents (e.g. ethyl acetate and diethyl ether) whereas DON and its parent compound nivalenol are soluble in polar solvents such as alcohols (Trenholm *et al.* 1986; Hussein & Brasel 2001). The trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with initiation, elongation and termination stages (Kumar *et al.* 2013).

DON is one of the most frequently found mycotoxins in cereal grains worldwide (Hooft *et al.* 2011). Common clinical symptoms of DON toxicity include reduced growth and feed intake, vomiting, diarrhoea, gastrointestinal haemorrhaging, inflammation and alteration of the immune response (Rotter *et al.* 1996; Pestka 2007). In aquaculture species, DON ingestion has been associated with highly significant decreases in growth, feed intake, feed efficiency and protein and energy utilization (Hooft *et al.* 2011).

Fumonisins

Fumonisins are characterized by having a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine), which plays a role in their toxicity (Wang *et al.* 1992). Fumonisins inhibits the sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase), a key enzyme in the lipid metabolism, resulting in a disruption of this pathway. This enzyme catalyses the acylation of sphinganine in the biosynthesis of sphingolipids and also the deacylation of dietary sphingosine and the sphingosine that is released by the degradation of complex sphingolipids (ceramide, sphingomyelin and glycosphingolipid) (Wang *et al.* 1991). Sphingolipids are important for the membrane and lipoprotein structure and also for cell regulations and communications (second messenger for growth factors) (Berg *et al.* 2014). They are mainly produced by a number of *Fusarium* species, notably *F. verticillioides* (formerly *F. moniliforme* = *Gibberella fujikuroi*), *F. proliferatum* and *F. nygamai*. The most abundantly produced mycotoxin of the *Fusarium* family is fumonisin B1 (FB1), shown to promote tumours in rats (Gelderblom *et al.* 1988), to cause equine leukoencephalomalacia (Marasas *et al.* 1988) and porcine pulmonary oedema (Harrison *et al.* 1990). In aquaculture species, fumonisin B1 has been associated with reduced growth rate, feed consumption and feed efficiency ratio and impaired sphingolipid metabolism (Goel *et al.* 1994; Li *et al.* 1994; Lumlertdacha *et al.* 1995; Tuan *et al.* 2003).

Ochratoxins

Ochratoxins are metabolites of both *Aspergillus* and *Penicillium* species, which are chemically described as 3,4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L-β-phenylalanine (Cole & Cox 1981). Ochratoxin A (OTA) is the most toxic compound of this group (Hussein & Brasel 2001). These compounds are known for their nephrotoxic and hepatotoxic effects in livestock (Lanza *et al.* 1980; Manning & Wyatt 1984). Indeed, OTA has already been traced in meat (Guillamont *et al.* 2005), milk (Skaug 1999) and dairy products (Dall'Asta *et al.* 2008), and other animal-derived swine products (Pozzo *et al.* 2010). In aquaculture species, the fact that OTA may cause severe degeneration and necrosis of kidney and liver leading to inferior weight gains, poorer feed conversion rate, lower survival and haematocrit (Doster *et al.* 1972; Lovell 1992; Manning *et al.* 2003) has been reported.

Occurrence of mycotoxins in aquafeeds

Materials and methods

Samples

Over a period of 1 year (January 2014–December 2014), 41 samples of finish aquaculture feed, both shrimp and fish, have been analysed within the scope of BIOMIN mycotoxin survey programme and 154 individual analyses have been conducted. The samples were tested for aflatoxins (sum of AFB1, AFB2, AFG1 and AFG2), zearalenone, deoxynivalenol, fumonisins (sum of FB1 and FB2) and ochratoxin A (full toxin screen).

Sample origin

The samples were sourced in Asia (31 samples) and Europe (10 samples). Of the Asian samples, 21 were sourced in northern Asia (NAS – Singapore (CN)), two in southern Asia (SAS – India (IN)) and eight in South-East Asia (SEA – Thailand (TH) and Myanmar (MM)). European samples originated primarily from southern Europe, six samples from Croatia (HR) and four samples from Portugal (PT). The samples were acquired directly from aquaculture farms or feed producers. All sample providers were instructed on good sampling procedures and advised to follow them, based on the regulations by the European Commission from 2006 for sampling for mycotoxins in food and the ISO regulations from 2005 for sampling animal feedstuffs. However, analytical personnel or laboratory staff was not present to oversee the sampling.

Sampling and analysis

Sample providers were instructed to follow good sampling procedures according to Richard (2000). The analyses were carried out as described by Binder *et al.* (2007). All samples were analysed with HPLC. For the purpose of data analysis, nondetect levels are based on the limits of detection (LOD) of the HPLC. For aflatoxins, AFB1, AFB2, AFG1 and AFG,

the detection limit are 0.3, 0.1, 0.1 and 0.1 $\mu g\ kg^{-1}$, respectively. For zearalenone, deoxynivalenol and ochratoxin A, detection limits are as follows: 10, 50 and 0.2 $\mu g\ kg^{-1}$, and for fumonisins, the detection limit is 25 $\mu g\ kg^{-1}$ for both FB1 and FB2.

Results and discussion

The results obtained in this survey programme are summarized in Table 1. Generally, DON was most prevalent mycotoxin, with 68% of the samples testing positive, followed closely by AF and ZEN (59% positive) and by OTA and FB, with 57% and 51% respectively. Concerning the contamination level, FB was the one found in higher concentration, with an average of 637 $\mu g\ kg^{-1}$ on the 21 samples contaminated with this mycotoxin and showing a considerable maximum concentration of 7534 $\mu g\ kg^{-1}$. An important finding in this survey was the level of mycotoxin co-occurrence, which was frequently observed in aquaculture feed samples. In total, 76% of the samples had more than one mycotoxin, only 17% of the samples were contaminated by one mycotoxin, and just 7% of the samples did not contain detectable levels of any of the five mycotoxins (Table 1). Analysing the mycotoxin occurrence by region, it was possible to observe a different distribution pattern when comparing the mycotoxin occurrence between Europe and Asia, likely due to the climate differences between the two. In Europe (Table 2), it was observed that AF and OTA, while present in 17% and 67% of the samples, respectively, had relatively low average concentrations, 0.43 and 1.53 $\mu g\ kg^{-1}$, respectively (Fig. 1). The occurrence of the same mycotoxins in Asia was relative higher in percentage (AF = 68% and OTA = 55%) and in contamination level (AF = 51.83 $\mu g\ kg^{-1}$ and OTA = 2.11 $\mu g\ kg^{-1}$). However regarding ZEN, DON and FB, the higher average contamination values were found in European samples (ZEN = 118.01 $\mu g\ kg^{-1}$, DON = 165.61 $\mu g\ kg^{-1}$ and FB = 3419.92 $\mu g\ kg^{-1}$) (Fig. 1).

A recent review on mycotoxins and their consequences in aquaculture was published (Anater *et al.* 2015), which highlights the importance of mycotoxins for aquaculture. However, important species and studies were not addressed in this work, for example effects of mycotoxins in shrimps (Ostrowski-Meissner *et al.* 1995; Trigo-Stockli *et al.* 2000; Burgos-Hernández *et al.* 2005; Supamattaya *et al.* 2005) or in other important economical species such as gibel carp, Carassius auratus gibelio (Huang *et al.* 2011), red drum, *Sciaenops ocellatusand*, (Zychowski *et al.* 2013), hybrid sturgeon, *Acipenser ruthenus* × *A. baeri*, (Rajeev Raghavan *et al.* 2011) and Beluga, *Huso huso* (Sepahdari *et al.* 2010). Nonetheless, the important question that remains to be answered is which species can be affected by the mycotoxin levels found on aquaculture finish feeds. It is documented that mycotoxins can cause adverse effects in several aquatic species. These effects vary greatly depending on a variety of factors including nutritional and health status prior to exposure, dose and duration of exposure, age and species but also infection route (Hooft *et al.* 2011). Moreover, in some of the literature found, the mycotoxin concentrations tested are sometimes unrealistically high compared to naturally occurring levels and not representative of the mycotoxin load found on plant raw materials for aquaculture feeds. In this review, we compare the results obtained through the mycotoxin survey with the available literature on fish/shrimp mycotoxicoses, highlighting the real risk that the mycotoxins represent for aquaculture.

Rainbow trout (*Oncorhynchus mykiss*) are known to be extremely sensitive to low levels of DON (Woodward *et al.* 1983; Hooft *et al.* 2011). Hooft *et al.* (2011), reported that low, graded levels of DON ranging from 300 to 2600 $\mu g\ kg^{-1}$ from naturally contaminated corn resulted

Table 1 Overall results of the mycotoxin survey

	Mycotoxins				
	AF	ZEN	DON	FB	OTA
Number samples tested (*n*)	37	37	37	41	37
Positive samples (*n*)	22	22	25	21	21
% Positive	59	59	68	51	57
Negative samples (*n*)	15	15	12	20	16
% Negative	41	41	32	49	43
Average of positive (μg kg−1)	49	71	162	637	2
Maximum (μg kg−1)	221	306	413	7534	5
Sample origin	CN	HR	CN	PT	MM
Average (μg kg−1)	29	42	109	326	1

Co-occurrence	Samples contaminated
<limit of detection	7%
1 mycotoxin	17%
More than 1 mycotoxin	76%

Table 2 Regional results of the mycotoxin survey

	Mycotoxins				
	AF	ZEN	DON	FB	OTA
Europe					
Number samples tested (*n*)	6	6	6	10	6
Positive samples (*n*)	1	4	4	3	4
% Positive	17	67	67	30	67
Negative samples (*n*)	5	2	2	7	2
% Negative	83	33	33	70	33
Asia					
Number samples tested (*n*)	31	31	31	31	31
Positive samples (*n*)	21	18	21	18	17
% Positive	68	58	68	58	55
Negative samples (*n*)	10	13	10	13	14
% Negative	32	42	32	42	45

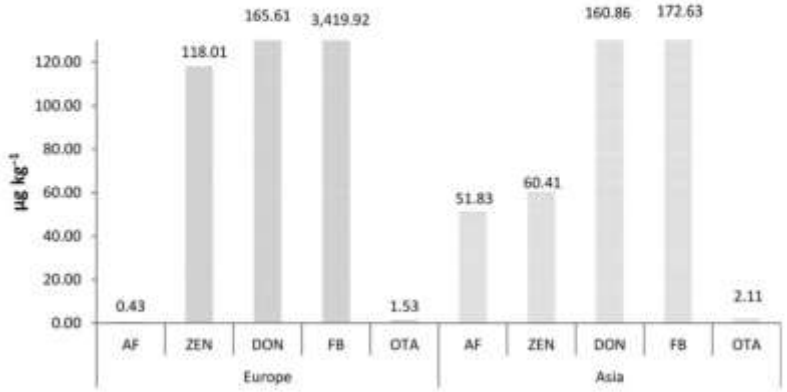

Figure 1 Average concentrations of aflatoxins (AF), ochratoxin A (OTA), zearalenone (ZEN), deoxynivalenol (DON) and fumonisins (FB) ($\mu g\ kg^{-1}$) in aquaculture finished feed in Europe and Asia.

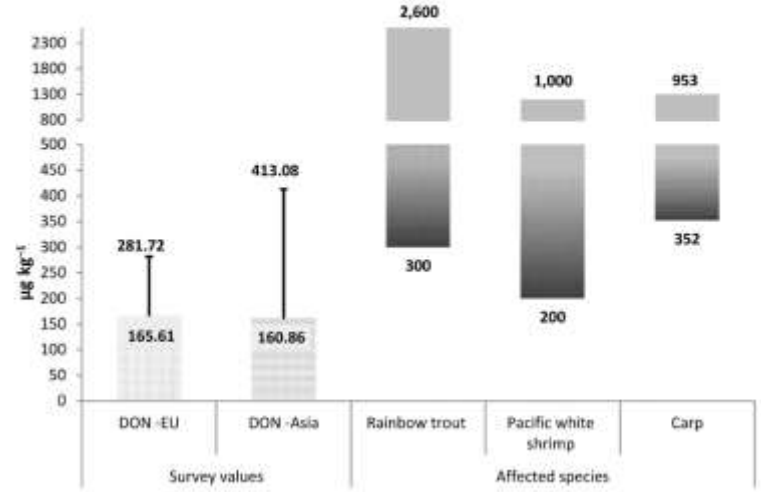

Figure 2 Comparison between DON values found in survey for Europe and Asia and species affected by these values. Survey values displayed as mean ± SE. Affected species values displayed as minimal and maximum values tested for the specie, based on the literature.

in highly significant decreases in growth (−40%), feed intake (−52.7%), feed efficiency (−76.7%) and protein and energy utilization (−74.4% and −72.1%) when compared to control. Another study with Atlantic salmon (*Salmo salar* L.) found that fish fed 3700 $\mu g\ kg^{-1}$ of DON had a 20% reduction in feed intake and a 31% decrease in specific growth rate (Döll *et al.* 2010). Contrarily, channel catfish (*Ictalurus punctatus*) fed diets containing up to 10 000 $\mu g\ kg^{-1}$ of DON from either a purified source or naturally contaminated wheat had no effects on feed consumption, growth, haematocrit values or liver weights (Manning *et al.* 2014). However, in Pacific white shrimp (*Litopenaeus vannamei*), DON levels ranging from 200 to 1000 $\mu g\ kg^{-1}$ in the diet significantly reduced shrimp body weight and/or growth rate (Trigo-Stockli *et al.* 2000). Also in carp (*Cyprinus carpio* L.), a feeding trial using three different concentrations of DON (352, 619 and 953 $\mu g\ kg^{-1}$) confirmed the immunosuppressive effects of DON even at the low doses (Pietsch *et al.* 2014). Directly comparing the previous literature values with contamination levels found in the survey (Fig. 2), we can observe that several of the species above described can be affected by DON in real aquaculture production scenarios. In European samples, an average value of 165.61 $\mu g\ kg^{-1}$ and maximum of 281.72 $\mu g\ kg^{-1}$ of DON was detected and in Asian samples an average value of 160.86 $\mu g\ kg^{-1}$ and maximum of

431.08 μg kg^{-1} of DON was found. These values are sufficient to affect rainbow trout, pacific white shrimp and carp production, according to the sensitive levels reported in literature. However, when making such comparisons, we are only considering a single mycotoxin effect. According to the survey data, mycotoxins co-occurrence for Europe was 50% and 84% for Asia (Figs 3,4). We would assume that the actual DON values of finished feed can affect more species than previously reported due to additive or synergistic effect with other mycotoxins present in the feed. Also, the tendency to substitute plant protein sources, mainly high protein diets, represents a potential threat for aquaculture and can cause a significant economic impact. Strong evidence suggests that at the survey levels, DON can have negative influences in growth and health performance but also disease susceptibility, due to ingestion of DON and possible other mycotoxins.

Aflatoxins were the most common mycotoxin found in the survey for Asia. Aflatoxins, mainly the toxicity of AFB1, has been considerable investigated in farmed fish and crustaceans species for aquaculture (Santacroce *et al.* 2008; Dirican 2015), including rainbow trout, *Oncorhynchus mykiss*

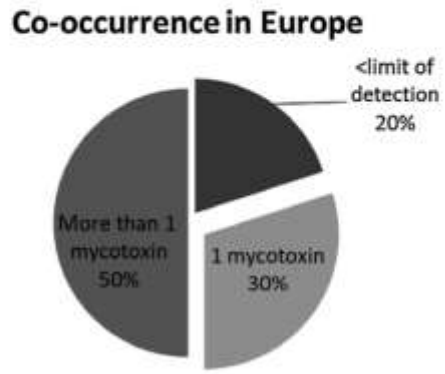

Figure 3 Percentage of mycotoxins co-occurrence in Europe.

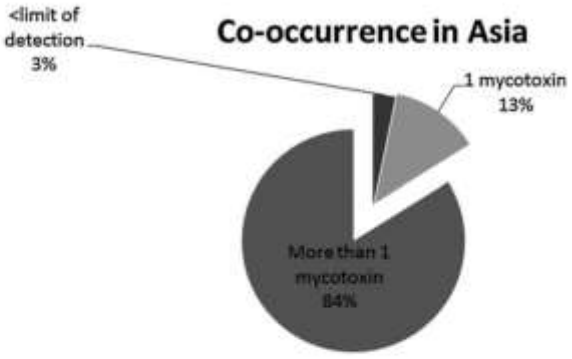

Figure 4 Percentage of mycotoxins co-occurrence in Asia.

(Halver 1969; Ngethe *et al.* 1992, 1993; Bailey *et al.* 1994; Takahashi *et al.* 1995; Ottinger & Kaattari 1998, 2000; Carlson *et al.* 2001; Hooft *et al.* 2011; Hanson *et al.* 2014; Arana *et al.* in press); channel catfish, *Ictalurus punctatus* (Plumb *et al.* 1986; Jantrarotai & Lovell 1990; Gallagher & Eaton 1995; Lumlertdacha *et al.* 1995; Manning *et al.* 2005) Nile tilapia, *Oreochromis niloticus*, (Chávez-Sánchez *et al.* 1994; Tuan *et al.* 2002; Deng *et al.* 2010; Hassan *et al.* 2010); rohu, *Labeo rohita* (Sahoo & Mukherjee 2001; Madhusudhanan *et al.* 2004; Ruby *et al.* 2013); seabass, *Dicentrarchus labrax* L. (El-Sayed & Khalil 2009; El-Sayed *et al.* 2009); gibel carp, *Carassius auratus gibelio* (Huang *et al.* 2011), red drum, *Sciaenops ocellatusand* (Zychowski *et al.* 2013); hybrid sturgeon, *Acipenser ruthenus* × *A. baeri* (Rajeev Raghavan *et al.* 2011); Beluga, *Huso huso* (Sepahdari *et al.* 2010); and Pacific white shrimp, *Litopenaeus vannamei* (Ostrowski-Meissner *et al.* 1995; Burgos-Hernández *et al.* 2005). As previously stated for DON, the biological effects of AF vary greatly over species and are also related to the toxin's concentration in feed and to animal's age and species (Hendricks 1994). On European species, it is reported that sea bass and rainbow trout are very sensitive to AF (LC50 = 180 μg kg^{-1} BW and 5–10 μg kg^{-1} BW, respectively) (Hendricks 1994; El-Sayed *et al.* 2009); however, the results obtained were by gavage feeding in the case of seabass and by intraperitoneal injection in trout. It could be difficult to extrapolate those findings for an AF contamination of feed, but evidence suggests that those species could be very sensitive to AF. Also Centoducati *et al.* (2010) concluded that Gilthead sea bream (*Sparus aurata*) hepatocytes are highly sensitive to AFB1 exposure, after exposing primary monolayer cultures of hepatocytes from *S. aurata* juveniles to a range of 5x10^{3} ng mL^{-1} to 2 × 10^{-5} ng mL^{-1} of AFB1 for a different period of exposure (24, 48 and 72 h). On Beluga, *Huso huso*, fed diets containing 25, 50, 75 and 100 μg of AFB1 kg^{-1} of diet for 3 months were negatively affected in terms of weight gain and FCR. A broad range of changes in liver tissue, including progressive fat deposition, hepatocyte degeneration and necrosis, particularly at concentration of 75 and 100 μg of AFB1 kg^{-1} of diet after 60 days were also recorded (Sepahdari *et al.* 2010). In another study, juvenile hybrid sturgeon, *Acipenser ruthenus* × *A. baeri*, fed diets containing graded levels of AFB1 (25–80 μg kg^{-1}), showed higher mortality in fish fed 80 μg kg^{-1} AFB1 from day 12 onwards despite no external changes or unusual behaviour being observed. Significant histopathological changes, including nuclear hypertrophy, hyperchromasia, extensive biliary hyperplasia, focal hepatocyte necrosis and the presence of inflammatory cells, were observed in the livers of fish fed high levels of aflatoxin (40 and 80 μg kg^{-1}). Furthermore, authors also found that AFB1 accumulation in fish muscle and liver enlarged with increased dietary AFB1

levels (Rajeev Raghavan *et al.* 2011). For tropical species, growth rate and FCR of Nile tilapia were reported to be significantly affected by AF on the feed. Values ranging from 100 to 2500 $\mu g\ kg^{-1}$ of AFB significantly affected growth performance in tilapia (El-Banna *et al.* 1992; Chávez-Sánchez *et al.* 1994; Tuan *et al.* 2003; Oliveira *et al.* 2013). It was also shown by El-Banna *et al.* (1992) that at lower concentrations of AF in the diets (50 $\mu g\ kg^{-1}$ of AFB), vacuolization and necrosis of hepatocytes were observed. Regarding mortality directly linked to AF ingestion, it was shown that animals fed diets containing AF as high as 30 000 $\mu g\ kg^{-1}$ of AFB for 25 days had no significant reduced survival (Chávez-Sánchez *et al.* 1994; Tuan *et al.* 2003). However, an earlier study reported that Nile tilapia fed a diet containing 200 $\mu g\ kg^{-1}$ of AFB for 10 weeks saw mortality increase by 16.7% (El-Banna *et al.* 1992). On rohu, *Labeo rohita*, AFB1 proved to be immunosuppressive at 1250 μg kg body weight of toxin (Sahoo & Mukherjee 2001); however, at lower dosages, 100 μg per 100 g BW, Madhusudhanan *et al.* (2004) found that AFB1 induces oxidative damage to the primary target organ, the liver, but also in the kidneys and brain. Nonetheless, it is important to mention that in previous studies made in rohu, an intraperitoneal administration route was used. On red drum (*Sciaenops ocellatus*), Zychowski *et al.* (2013) reported the species is susceptible to AFB1 in levels as low as 0.1 $\mu g\ kg^{-1}$. Authors showed that AFB1 negatively impacted red drum weight gain, survival, feed efficiency, serum lysozyme concentration, hepatosomatic index (HSI), whole-body lipid levels, liver histopathological scoring and trypsin inhibition. Huang *et al.* (2011) reported that gibel carp (*Carassius gibelio*) is a species less susceptible to AFB1. During 12 weeks of exposure up to approximately 1000 μg AFB1 kg^{-1} of feed, fish were not affected. The authors also reported a strong clearance ability of AFB1 during recovery period. Channel catfish were also reported to be relatively resistant to AFB1 when compared with other species (Jantrarotai & Lovell 1990). Previously, authors reported that gross appearance and behaviour of all fish were normal after being fed 10 000 μg AFB1 kg^{-1} of feed for 10 weeks. However, after 10 weeks, histopathological effects were observed on fish and gastric glands in the stomach were necrotic and contained infiltrating macrophages. For shrimp, Wiseman *et al.* (1982) reported that an injection of AFB1 into the tail muscle of Pacific blue shrimp (*Penaeus stylirostri*) produced 24- and 96-h median lethal doses of 100.5 and 49.5 mg kg^{-1} of AFB1, respectively. Although using IM injection, it seems that shrimp could also be very sensitive to aflatoxins. Regarding *in vivo* studies, in black tiger shrimp (*Penaeus monodon Fabricius*) fed AFB1 levels ranging from 5 to 20 $\mu g\ kg^{-1}$ had an decreased of 46–59% of the body weight on the AFB1-treated groups compared to control (Bintvihok *et al.* 2003). Also in black tiger shrimp, Boonyaratpalin *et al.* (2001) observed that AFB1 ranging from 50 to 100 $\mu g\ kg^{-1}$ did not affect growth performance. However, growth was reduced when aflatoxin B1 concentrations were elevated to 500 and 2500 $\mu g\ kg^{-1}$. The author observed that survival dropped to 26.32% when 2500 $\mu g\ kg^{-1}$ of AFB1 was given, whereas concentrations of 50–1000 $\mu g\ kg^{-1}$ had no effect on survival. On Pacific white shrimp, Ostrowski-Meissner *et al.* (1995) reported that abnormal hepatopancreas and antennal gland tissues were caused by 2 weeks of AFB1 at 50 $\mu g\ kg^{-1}$, feed conversion and growth were significantly affected at 400 $\mu g\ kg^{-1}$ AFB1, and apparent digestibility coefficients decreased significantly at 900 $\mu g\ kg^{-1}$ AFB1. The contamination values of AF found for Europe were negligible (0.43 $\mu g\ kg^{-1}$); however, the values found for Asia (average = 51.83 $\mu g\ kg^{-1}$ and maximum of 220.61 $\mu g\ kg^{-1}$; Fig. 1) can impact several rearing species (Fig. 5). It is important to emphasize that mycotoxin co-occurrence was very high and the additive/synergistic effects are not being taking into account in this comparison. Also, the global trade in raw materials and aquaculture feeds could potentially export the occurrence of mycotoxins to other regions.

Regarding ZEN, one of the most prevalence mycotoxins in Europe, it has mostly been studied for its ability to cause disorders in the reproductive tract of farm animals (Zinedine *et al.* 2007; Minervini & Aquila 2008). However, the effect of zearalenone on fish and shrimp has hardly been evaluated. The few studies existing show that ZEN can modulate ER-dependent gene expression affecting the reproduction of fish. This has been shown in zebrafish (*Danio rerio*) where the exposure to ZEN reduced spawning frequency (Schwartz *et al.* 2010) or changed their relative fecundity from one generation to another (Schwartz *et al.* 2013). In other study, when zebrafish larvae were exposed to 500 $\mu g\ L^{-1}$ or higher of ZEN, defects in heart and eye development and upward curvature of the body axis were observed (Bakos *et al.* 2013). Among aquaculture-farmed species, when black tiger shrimp (*Penaeus monodon Fabricius*) were fed 500 and 1000 $\mu g\ kg^{-1}$ ZEN-contaminated feed, histological changes were observed in hepatopancreatic tissue (Supamattaya *et al.* 2005). For carp (*Cyprinus carpio* L.), Pietsch *et al.* (2015) investigated ZEN at three different concentrations (332, 621 and 797 $\mu g\ kg^{-1}$) for 4 weeks. The authors observed no effect on growth, but effects on haematological parameters were confirmed. In addition, an influence on white blood cell counts was noted whereby granulocytes and monocytes were affected in fish fed 621 and 797 $\mu g\ kg^{-1}$ of ZEN in the diet. Furthermore, marginal ZEN and α-zearalenol (α-ZEL) concentrations were detected in muscle samples and the genotoxic potential of ZEN was confirmed by analyzing formation of

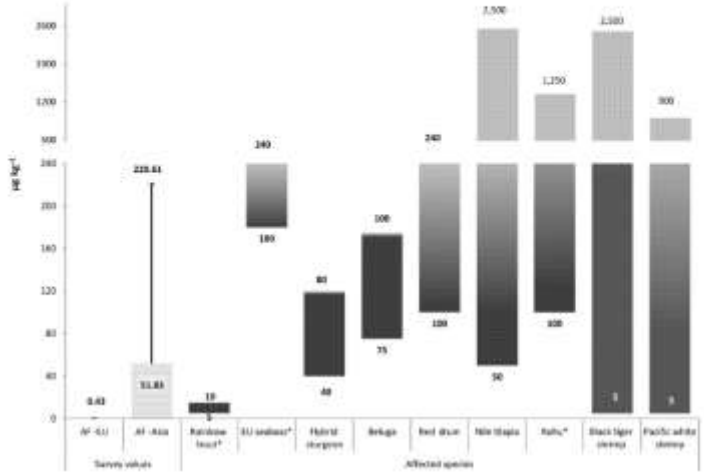

Figure 5 Comparison between AF values found in survey for Europe and Asia and species affected by these values. Survey values displayed as mean ± SE. Affected species values displayed as minimal and maximum values tested for the specie, based on the literature. *Values are reported in μg kg^{-1} BW.

micronuclei in erythrocytes. In juvenile rainbow trout (*Oncorhynchus mykiss*), Woźny *et al.* (2012) observed that after 24, 72 and 168 h of intraperitoneal exposure of 10 000 μg of ZEN kg^{-1} of body weight, the mycotoxin tested was able to interfere with blood coagulation and iron storage processes. However, in another study, Woźny *et al.* (2015) observed that trout fed ZEN-contaminated feed at a dose of 1810 μg kg^{-1} had no effects on growth, but results suggested that ZEN may accelerate sexual maturation of female fish. The values of ZEN found for Europe and Asia were lower than the reported sensibility values of aquaculture species. This does not necessarily mean that aquaculture species are resistant to ZEN. Rather, it highlights the lack of scientific studies in this area and the consequent difficulty in understanding the risk that this mycotoxin represents to aquaculture. However, taking into consideration the zebrafish studies and the few studies made in aquaculture species, we would assume that ingestion of ZEN may affect growth performance and disease susceptibility following short-term exposure, and over a longer term, it may lead to complications in broodstocks of farmed species and monosex-cultured species.

Scarce information is also available on the effects of fumonisins on aquaculture species. In channel catfish, dietary levels of FB1 at or above 20 000 μg kg^{-1} have shown to be toxic (Lumlertdacha *et al.* 1995). It is also known that rainbow trout liver is sensitive to FB, inducing changes in sphingolipid metabolism on values lower than 100 μg kg^{-1} (Meredith *et al.* 1998) and inducing cancer in 1-month-old trout (Riley *et al.* 2001). Tuan *et al.* (2003) demonstrated that feeding FB1 at levels of 10, 40, 70 and 150 mg kg^{-1} feed for 8 weeks affected growth performance of Nile tilapia fingerlings. In this last experiment, fish fed diets containing FB1 at levels of 40 000 μg kg^{-1} or higher had decreased average weight gains. Haematocrit was decreased only in tilapia fed diets containing 150 000 μg FB1 kg^{-1}. The ratio between free sphinganine and free sphingosine (Sa:So ratio) in liver increased at 150 000 μg FB1 kg^{-1} in the fish feed. Adverse effects of fumonisin-contaminated diets have also been reported in carp. One-year-old carp showed signs of toxicity with 10 000 μg FB1 kg^{-1} feed (Petrinec *et al.* 2004). In these experiments, scattered lesions in the exocrine and endocrine pancreas and interrenal tissue, probably due to ischaemia and/or increased endothelial permeability, were reported. In another study, 1-year-old carp consumed pellets contaminated with 500 and 5000 150 000 μg FB1 kg^{-1} of body weight, resulting in a loss of body weight and alterations of haematological and biochemical parameters in target organs (Pepeljnjak *et al.* 2003). In Baltic salmon (*Salmon salar*), it was observed that animals fed graded levels of FB1 from 1000, 5000, 10 000 or 20 000 μg kg^{-1} of feed for 10 weeks appeared unaffected in terms of growth, feed intake and liver damage (García 2013). However, during the experimental period, it was observed that all fish (including control group) had very poor appetite and grew very slowly when compared to SGR values 2–6 times higher reported in other studies (Farmer *et al.* 1983; McCormick *et al.* 1998). On the previous thesis, the values of FB1 on the feed after extrusion were not reported, leading us to assume that real inclusion levels of FB1 on the feed might be different from the ones theoretical projected.

Fumonisin B1 has not been extensively studied as a shrimp feed contaminant; however, the few studies

available suggest that *Litopenaeus vannamei* is sensitive to FB1. García-Morales *et al.* (2013) have shown that white shrimp fed FB1 at levels from 20 to 200 $\mu g\ kg^{-1}$ had a reduction in soluble muscle protein concentration and changes in myosin thermodynamic properties were observed in shrimp after 30 days of exposure to FB. The same authors reported marked histological changes in tissue of shrimp fed a diet containing FB1 at 200 $\mu g\ kg^{-1}$ and meat quality changes, after 12 days of ice storage, when fed diets containing more than 600 $\mu g\ kg^{-1}$ FB. When comparing literature sensitive levels for aquaculture species and the contamination values found in the survey, we observe that several species can be affected by FB (Fig. 6). The contamination levels reported are again sufficient to harm several aquaculture species, assuming single mycotoxin contamination. In the case of Europe, the values found are alarmingly dangerous. The prevalence of this mycotoxin in Europe was relatively low (30%); however, it was detected at high levels (average value of 3419.92 $\mu g\ kg^{-1}$; maximum value of 7533.61 $\mu g\ kg^{-1}$). At this level, FB represents a serious risk for aquaculture production in Europe, being on average, 10 times higher than EU guidance values given for FB1 (10 $\mu g\ kg^{-1}$ for complementary and complete feeding stuffs for fish). These figures lead us to expect severe consequences for the aquaculture industry and mainly for carnivorous species. Further studies on the effects of FB in European species are urgently needed.

Studies on the toxicity of OTA in aquatic animals are very scarce. Severe abnormalities such as deformities of the head, tail and eyes were found in Zebrafish, after the developing eggs were exposed to OTA (Debeaupuis *et al.* 1984). In rainbow trout intoxicated with OTA (Doster *et al.* 1972), severe degeneration and necrosis of kidney and liver, pale kidney, light swollen livers and mortality occurred. The author found an LD_{50} for OTA of 5.53 mg kg^{-1} body weight (13-72 $\mu mols\ kg^{-1}$). On channel catfish (*Ictalurus punctatus*) fed 5, 1.0, 2.0, 4.0, or 8.0 mg OA kg^{-1} diets, authors found significant reduction in weight gains, poorer feed conversion rate, lower survival and haematocrit. Moreover, moderate-to-severe histopathological lesions of liver and posterior kidney were observed (Lovell 1992; Manning *et al.* 2003). For common carp (*Cyprinus carpio*), Agouz and Anwer (2011) showed that a natural contamination of 15 $\mu g\ kg^{-1}$ of OTA in the diet resulted in decreasing growth performance and feed utilization parameters. Carcass dry matter, protein and ash contents are negatively correlated with OTA. In European seabass (*Dicentrarchus labrax* L.), El-Sayed *et al.*(2009) found the 96 h LC_{50} value at 277 $\mu g\ kg^{-1}$ bwt with 95% confidence limits of 244–311 $\mu g\ kg^{-1}$ bw. Nevertheless, Supamattaya *et al.* (2005) noticed that shrimp feeds occasionally contaminated with OTA (~1000 $\mu g\ kg^{-1}$) have no negative impact on the shrimp culture industry. The occurrence of OTA in Europe and Asia is quite high, at 67% and 55%, respectively, although the contamination value is relatively low (1.02 and 1.15 $\mu g\ kg^{-1}$, respectively). These figures are not indicative of a direct risk for the aforementioned species when considering single mycotoxin contamination. Nevertheless synergistic effects with other mycotoxins should be considered even at low levels.

Mycotoxins co-occurrence and synergistic effects of mycotoxins

Considering the fact that compound feed contains a mixture of several raw materials and adding to this, the fact that mycotoxigenic fungi are usually capable of producing more than one mycotoxin is not a surprise to observe a mycotoxin co-occurrence of 76% on the samples collected (Table 1). In Europe, 50% of the samples had more than 1 mycotoxin per sample (Fig. 2), and in Asia, 84% of the samples were contaminated by more than 1 toxin per feed sample (Fig. 3). Registers of mycotoxin co-occurrence in fish feeds are documented in the past for Egypt

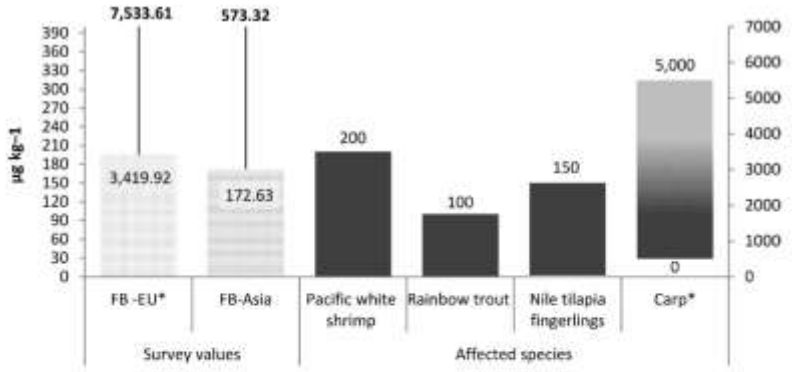

Figure 6 Comparison between FB values found in survey for Europe and Asia and species affected by these values. Survey values displayed as mean ± SE. Affected species values displayed as minimal and maximum values tested for the specie, based on the literature. *Values are plotted on secondary axis.

(Abdelhamid *et al.* 1998), United States (Lumlertdacha & Lovell 1995), Indonesia (Ali *et al.* 1998), Nigeria (Omodu Foluke Olorunfemi *et al.* 2013), Central Europe and Switzerland (Pietsch *et al.* 2013) and Brasil (Barbosa *et al.* 2013a). The present survey and previous literature elucidates the importance of this topic in aquaculture. It is known that mycotoxin multi-exposure may lead to additive, synergistic or antagonist toxic effects (Alassane-Kpembi *et al.* 2013) and Brasil (Barbosa *et al.* 2013a). However, the scarce data on toxic effects of mycotoxins mixtures restrict our knowledge on the real risk from multimycotoxin exposure. Indeed, there are few studies addressing the combined effects of mycotoxins in livestock (Kubena *et al.* 1997; Morris *et al.* 1999) and even fewer in aquaculture (Carlson *et al.* 2001). Carlson *et al.* (2001) showed in rainbow trout that FB1 was not carcinogenic when fed at 0, 3.2, 23 or 104 mg kg^{-1} FB1 for a total of 34 weeks. However, trout fed FB1 (≥23 mg kg^{-1} FB1 for 42 weeks) promoted AFB1-initiated liver tumours; this result also highlights the importance of long-term contamination as a factor influencing the susceptibility of animals. The anterior author also suggested that the FB1 promotional activity in AFB1-initiated fish was correlated with disruption of sphingolipid metabolism, suggesting that alterations in associated sphingolipid signalling pathways are potentially responsible for the promotional activity of FB1 in AFB1-initiated fish. McKean *et al.* (2006c) studied the combined effects of aflatoxin B1 and T-2 toxin in mosquitofish (*Gambusia affinis*), showing a significant additive interaction in the toxic response to the combination of mycotoxins. On carp (*Cyprinus carpio*), He *et al.* (2010) studied the individual and combined effects of DON and AFB1 on primary hepatocytes and concluded that the toxic effects of the combined mycotoxins were bigger than the effects of single mycotoxins. To our knowledge, no other studies were carried out for exploring the interacting effects of multimycotoxins in aquaculture-farmed animals; however; taking into consideration the studies carried out for other species (Segvic Klaric 2012), we could consider that mycotoxins interactions may also have negative effects on fish and shrimp. For example, in swine, AFB1 and OTA had additive interactions according to liver weight and blood chemistry, but they were antagonists with regard to the degree of renal cortical interstitial fibrosis and relative kidney weight (Harvey *et al.* 1995). In rats, AFB1 and OTA showed no interaction regarding the measurement of mortality, weight gain or most serum biological parameters, but the anaplastic and hyperchromatic nuclei, necrosis and bile duct proliferation observed were more pronounced in the combined toxin group after 4 months (Rati *et al.*, 1991). *In vitro* studies performed on human cell lines show thesynergic effects between AFB1 + T-2 toxin (T-2) and AFB1 + fumonisin B1 (FB1) in BEAS-2B (human bronchus epithelial cell line), while there was only an additive effect in AFB1 + T-2 mixtures and a slight antagonism between AFB1-FB1 in Hep G2 (human hepatocarcinoma cell line) (McKean *et al.* 2006a,b). OTA and FB1 were also tested together in Caco-2 (human colorectal adenocarcinoma) and Vero (green monkey renal) cell lines. The couple yielded a synergic behaviour, possibly due to their ROS production capability (Creppy *et al.* 2004). With regard to AFB1 and OTA, AFB1 was found to be mutagenic with metabolic activation and OTA was not mutagenic. Mutagenic capacity was assessed by an Ames test, if the mutagenic potential of mycotoxins compounds were analysed. A positive test indicates that the toxin is mutagenic and therefore may act as a carcinogen, because cancer is often linked to mutation. Even though the OTA increased the mutagenicity of AFB1 and the mixture not only showed cytotoxic additive effects but also a slight increase in DNA fragmentation as compared to mycotoxins taken separately in Vero cells (Golli-Bennour *et al.* 2010).

Carry-over of mycotoxins

Information about the carry-over of mycotoxins from feed ingredients and feed to animal food products is an important issue (Leeman *et al.* 2007). This topic has been address by the European Food Safety Authorities (EFSA) for carry-over of several mycotoxins in terrestrial animals such as poultry, swine and cow (EFSA, 2004), while no information exists on the carry-over in farmed fish species. Recently, Nácher-Mestre *et al.* (2015) evaluated the potential carry-over of mycotoxins from feed to gilthead sea bream and Atlantic salmon meat. The authors did not detect mycotoxins in fish tissues after feeding sea bream and Atlantic salmon for 8 and 7 months, respectively. However, important findings were observed. First, the authors observed co-occurrence of mycotoxins in four of the seven plant ingredients used. In the case of corn, the co-occurrence of fumonisins B1, B2 and B3, DON, 15-AcDON, HT2, T-2, ZEN and OTA were found. Second, the authors observed that in certain cases, the values of mycotoxins found in the diets were higher than expected taking into account the ingredients' sources contaminations levels, concluding that fungi grow heterogeneously when stored (Nácher-Mestre *et al.* 2015).

Survey limitations

Authors are aware that such mycotoxin survey faces some limitations due to its sampling complexity and representativeness. Some samples analysed for Asia and Europe are limited in their number. However, as preliminary approach to understand how aquaculture diets are being affected by mycotoxins, mostly by the inclusion of plant nutrients, this survey highlights several aspects that are important to consider. Finished feed samples were collected from the most

Table 3 Documented mycotoxins effects on aquaculture species and mostly contaminating feedstuffs

Mycotoxins/Fungus species	Documented effects on aquaculture species	Mostly contaminating feedstuffs†	Studied species/tested dosage	Key references
B-Trichothecenes (Nivalenol, deoxynivalenol (DON), fusarenon-x) *Fusarium* sp. *F. poae* *F. sporotrichioides* *F. equiseti* *F. crookwellense* *F. acuminatum* *F. sambucinum*	DON can cause feed refusal and reduced feed efficiency. Possible effects on fish/shrimp: • Reduced growth; reduced feed consumption; poor FCR • Lower weight gain; higher mortality • Heterogeneous growth; physiological disorders • Lower haematocrit values (reduced number and size of red blood cells) • Decreased resistance to environmental and microbial stressors • Increased susceptibility to diseases	High prevalence in cereal grains around the world. Common on: • Corn • Wheat • Barley • Rice bran • Corn • Soy bean meal • Seed meal • Peanut meal	Rainbow trout (*Oncorhynchus mykiss*) 300–2600 $\mu g\ kg^{-1}$ Atlantic salmon (*Salmo salar* L.) 3700 $\mu g\ kg^{-1}$ Channel catfish (*Ictalurus punctatus*) 10 000 $\mu g\ kg^{-1}$ Pacific white shrimp (*Litopenaeus vannamei*) 200–1000 $\mu g\ kg^{-1}$ Common carp (*Cyprinus carpio* L.) 352–953 $\mu g\ kg^{-1}$	Hooft *et al.* (2011) Döll *et al.* (2010) Manning *et al.* (2014) Trigo-Stockli *et al.* (2000) Pietsch *et al.* (2014)
Aflatoxins (B1, B2, G1, G2) *Aspergillus* spp. *A. flavus* *A. parasiticus* *A. nomius* *A. pseudotamarii* *A. bombycis* *A. ochraceoroseus* *A. nomius* *A. pseudotamari*	Aflatoxins are highly carcinogenic. Possible effects on fish/shrimp: • Higher incidence of cancer in exposed animals; liver tumours • Reduced growth; low apparent digestibility; higher mortality • Severe hepatic necrosis; liver lesions • Pale gills • Negative effect on digestive enzymes • Impaired blood clotting; poor growth rates • Yellow eyes, yellowed mucous membranes or skin • Lower hematocrit value (reduced number and size of red blood cells)	Commonly found in high starch and lipid content raw materials, such as: • Cottonseed meal • Peanut meal • Corn • Corn gluten • Soybean • Maize • Rice • Sunflower meal • Cassava • Palm kernel meal • Wheat	Rainbow trout (*Oncorhynchus mykiss*) LC50 = 5–10 $\mu g\ kg^{-1}$ BW European seabass (*Dicentrarchus labrax* L.) LC50 = 180 $\mu g\ kg^{-1}$ BW Beluga (*Huso huso*) 75 and 100 ppb AFB1 kg^{-1} Hybrid sturgeon (*Acipenser ruthenus* × *A. baeri*) 25–80 $\mu g\ kg^{-1}$ Nile tilapia (*Oreochromis niloticus*) 50–2500 $\mu g\ kg^{-1}$ Rohu (*Labeo rohita*) 100–1250 $\mu g\ kg^{-1}$ BW Red Drum (*Sciaenops ocellatus*) 0.1 $\mu g\ kg^{-1}$ Gibel carp (*Carassius gibelio*) 1000 $\mu g\ kg^{-1}$ Channel catfish (*Ictalurus punctatus*) 10 000 $\mu g\ kg^{-1}$ Pacific blue shrimp (*Penaeus stylirostri*) 24 and 96h LC50 of 100.5 and 49.5 mg kg^{-1} Black tiger shrimp (*Penaeus monodon Fabricius*) 5–2500 $\mu g\ kg^{-1}$ Pacific white shrimp (*Litopenaeus vannamei*) 50–900 $\mu g\ kg^{-1}$	Hendricks (1994) El-Sayed and Khalil (2009) Sepahdari *et al.* (2010) Rajeev Raghavan *et al.* (2011) El-Banna *et al.* (1992), Tuan *et al.* (2002) Sahoo and Mukherjee (2001), Madhusudhanan *et al.* (2004) Zychowski *et al.* (2013) Huang *et al.* (2011) Jantrarotai and Lovell (1990) Ostrowski-Meissner *et al.* (1995) Boonyaratpalin *et al.* (2001), Bintvihok *et al.* (2003) Ostrowski-Meissner et al. (1995)

Table 3 (continued)

Mycotoxins/Fungus species	Documented effects on aquaculture species	Mostly contaminating feedstuffs†	Studied species/tested dosage	Key references
Zearalenone *Fusarium* spp. *F. culmorum* *F. graminearum* *F. sporotrichioides*	ZEN shows estrogenic properties causing reproductive disorders. Possible effects on fish/shrimp: Zearalenone has been shown to cause reduced growth in white shrimp. It can also be deposited in the meat.	All fungus species are regular contaminants of cereal crops worldwide, such as: • Cottonseed meal • Peanut meal • Seed meal • Corn gluten • Cassava • Palm kernel meal • Corn gluten • Fish meal	Black tiger shrimp black (*Penaeus monodon Fabricius*) 500–1000 $\mu g\ kg^{-1}$ Common Carp (*Cyprinus carpio* L.) 621–797 $\mu g\ kg^{-1}$ Rainbow trout (*Oncorhynchus mykiss*) 1810–10 000 $\mu g\ kg^{-1}$	Supamattaya *et al.* (2005) Pietsch *et al.* (2015) Woźny *et al.* (2015)
Fumonisins (Fumonisin B1, B2, B3, fusaric acid) *Fusarium* spp. *F. verticillioides* *F. proliferatum* *F. nygamai*	FUM primarily target organs are the kidneys and the liver. Possible effects on fish/shrimp: • Lower hematocrit values • Lesions in the exocrine and endocrine pancreas • Lesions in the inter-renal tissue • Changes in myosin thermodynamic properties of muscle • Reduced growth rate • Reduced feed consumption • Impaired sphingolipid metabolism	Commonly found in raw materials, such as: • Corn • Maize flour • Seed meal • Wheat	Channel catfish (*Ictalurus punctatus*) at or above 20 000 $\mu g\ kg^{-1}$ Rainbow trout (*Oncorhynchus mykiss*) 100 $\mu g\ kg^{-1}$ Nile tilapia (*Oreochromis niloticus*) 10–150 mg kg^{-1} Common Carp (*Cyprinus carpio* L.) with 500–150 000 $\mu g\ kg^{-1}$ Pacific white shrimp (*Litopenaeus vannamei*) from 20 to 200 $\mu g\ kg^{-1}$	Lumlertdacha *et al.* (1995) Meredith *et al.* (1998) Tuan *et al.* (2003) Pepeljnjak *et al.* (2003), Petrinec *et al.* (2004) García-Morales *et al.* (2013)
Ochratoxin A (ochratoxin A) *Aspergillus* spp. *A. ochraceus* *A. alliaceus* *A. auricomus* *A. carbonarius* *A. glaucus* *Penicillium* spp. *P. verrucosum* *P. viridicatum*	OTA cause histopathological lesions of the liver Possible effects on fish/shrimp: • Reduced growth; higher mortality; poor FCR; reduced body weight • Liver and kidney necrosis • Pale, swollen kidneys • Severe abnormalities such as deformities of the head, tail and eyes	Commonly found in high starch and lipid content raw materials, such as: • Cottonseed meal • Sunflower meal • Peanut meal • Soy bean meal • Corn • Palm kernel meal	Channel catfish (*Ictalurus punctatus*) 5–8.0 mg OA kg^{-1} diet Common carp (*Cyprinus carpio*) 15 $\mu g\ kg^{-1}$ European seabass (*Dicentrarchus labrax* L.) 96 h LC50 value at 277 $\mu g\ kg^{-1}$ bwt Rainbow trout (*Oncorhynchus mykiss*) LD50 of 5.53 mg kg^{-1} body weight (13.72 $\mu mols\ kg^{-1}$)	Lovell (1992), Manning *et al.* (2003) Agouz and Anwer (2011) El-Sayed *et al.* (2009) Doster *et al.* (1972)

†The mostly contaminating feedstuffs is based on Spring & Fegan (2005), Ostrowski-Meissner *et al.* (1995) and Hussein & Brasel (2001).

representative companies in each region and sampled from the current feed batches produced. In this way, we guarantee that the feeds analysed were actually used to feed animals under normal rearing conditions. Finished feeds were not analysed or selected by/for plant ingredients, that is the samples analysed could had or not plant material on its composition; however, this was not accessed. This guaranteed that samples were the most representative feeds being used for aquaculture in these regions, independently of its formulation. Several feed companies have their own mycotoxin screening programmes; however, to our knowledge, due to time and cost limitations, screenings are not performed for all commodities purchased nor for all finished feeds. Mycotoxins screens are mostly used as a tool to outwit suspicious or frequently contaminated commodities. The occurrence of mycotoxins varies greatly according to different factors (e.g. temperature, type of raw material, geographical location). We would also need to assume the possibility of toxin production during storage of commodities and/or finished feeds.

Conclusion

With the increasing substitution of fishmeal with plant feed ingredients in aquaculture feeds, the risk of mycotoxin contamination is real, as demonstrated by the present work (Table 3). The small amount of existing literature on the topic is sometimes inconsistent even for the same species, making it difficult to draw firm conclusions. This could be due to factors as follows: age, nutritional and health status, rearing densities and environmental conditions of animals studied. However, we have to consider that the duration of experiments, most of the experiments last for 8–12 weeks, and during that time, we observe that different performance parameters are affected, being mortality only affected at the end of the experimental period, probably as a consequence of severe tissue damage. Another important factor to have into consideration is that animal experimentation trials are normally performed under optimal rearing conditions. We could assume that under real farming conditions, animals would be more sensitive to mycotoxins when compared to experimental values. Another difficulty when dealing with mycotoxins in aquaculture is that clinical signs are mainly subclinical and hard to detect, being easily the cause of death attributed to other cause instead of mycotoxins. This makes it more difficult to identify the full extent of the mycotoxin problem and also decreases the possibility of react to pathological evidences. The best approach to avoid losses in aquaculture industry should be active monitoring of feed for mycotoxins and implementation of a robust mycotoxin risk management programme.

The levels of mycotoxins detected in the survey, with the exception of FB1, were inferior to EU guidance values, and in some of the cases, below the sensitive levels reported in scientific literature for aquaculture farmed species. This does not necessary mean that those species or other species not mentioned in the review are not affected by mycotoxins. In some cases, there is lack of knowledge regarding mycotoxins in aquaculture, and in other cases, the levels tested are too high. Another limitation when comparing the contamination values found in the survey with the existing literature is the fact of basing the comparison on single mycotoxin effects. As we saw, the rate of co-occurrence is high and the knowledge on the effects of multiple mycotoxins contamination in aquatic species is relatively unknown. However, taking into consideration the studies made in livestock species, we would assume that the present contamination values found in aquaculture feed can represent a threat for aquaculture production. Still, regarding the FB1 detected for Europe, we would assume that this extremely high value can be seen as an exception that can be explained by poor storage conditions or even an unusually high raw material contamination. We would highlight the urgent need for frequent mycotoxin monitoring in aquaculture feed industry. As mycotoxins are also produced during storage conditions, it will be important to analyse not just raw materials but also finished feeds. Given the global trade in raw materials and aquaculture feeds and the impact of climate change on agriculture, it is difficult to predict mycotoxin occurrence by region. We would encourage a screening programme for the main mycotoxins affecting aquaculture production to be implemented for aquaculture raw materials and finished feeds.

An important issue that also deserves attention is the potential carry-over of mycotoxins from feed ingredients and finished feed to the meat of aquatic-farmed animals for human consumption. Taking into account the use of plant ingredients in aquaculture feeds, especially as a fish meal replacement for carnivorous species, we consider that more research is needed on the effect of mycotoxins in animal health but also on carry-over effects.

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Mycotoxins in aquaculture: feed and food

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Abstract

Mycotoxins, secondary metabolites produced by moulds, are responsible for causing significant economic losses due to spoilage of agricultural products but also due to direct or indirect health impact on livestock upon ingestion of mycotoxin contaminated feedstuffs. Aquaculture farmed species are not an exception and studies reporting mycotoxin-related issues in the aquaculture industry have been increasing. However, our understanding on the prevalence and impact of mycotoxins in the aquaculture sector is still lower compared to the terrestrial livestock sector. Consequently, regulatory limits and guidance values have been defined based on the studies on terrestrial farm animals. The aim of this review is to compile and critically assess mycotoxin occurrence and co-occurrence in aquaculture finished feeds, and understand the risk of mycotoxin carry-over in aquaculture seafood products. Furthermore, we aim with this review to raise awareness to the scientific community, the regulatory authorities and the aquaculture industry to the need for specific aquaculture mycotoxin maximum concentration levels for both aquaculture feeds and foods.

Key words: mycotoxins occurrence, carry-over effects, fish, shrimp, aquafeeds, transfer factor.

Introduction

Mycotoxins are secondary metabolites produced by some moulds (Hussein & Brasel 2001). These can be produced on agricultural commodities pre- and/or post-harvest including directly in finished feeds. Mycotoxins are responsible for significant economic losses due to the spoilage of agricultural products (Shane & Eaton 1994; Vasanthi & Bhat 1998; CAST 2003). Furthermore, mycotoxins can cause diseases problems when consumed by humans and livestock, causing significant problems worldwide (Zain 2011). Despite being identified as categorically undesirable for most aquaculture species, their occurrence, at least in field conditions, is not completely preventable even when using good manufacturing practices (FAO 1979). The awareness of mycotoxin-related issues in the aquaculture industry has been increasing, accentuated by the increased inclusion levels of plant meals in aquafeeds (Tacon *et al.* 2011). Traditionally, the use of minor amounts of plant feed stuffs led to an accepted perception that mycotoxins were not a relevant issue in aquaculture and that the majority of mycotoxin issues would stemmed only due to poor storage conditions. *Aspergillus* spp. and *Penicillium* spp. can grow on feed stored in poor conditions, ultimately leading to the production of aflatoxin (AF) and ochratoxin A (OTA). This would seem to be particularly the case in countries where climate conditions are favourable to the growth of *Aspergillus* spp. and *Penicillium* spp. fungi. However, optimal storage conditions should prevent the contamination of raw materials and finished feeds from AF or OTA. However, some plant commodities such as cottonseed and peanut meals commonly present detectable levels of AF and/or OTA (Gonçalves *et al.* 2017), even when stored using appropriate conditions.

With the increased use of plant meals in aquafeeds, other mycotoxins besides AF and OTA have been reported in finished feeds, as mycotoxins are reasonably stable to processing conditions (Cheli *et al.* 2013). *Fusarium* mycotoxins (Type B and A, trichothecenes and fumonisins) are, contrary to AF and OTA, mainly produced at pre-harvest stage. The production of these mycotoxins by *Fusarium* spp. seems to be highly influenced by environmental conditions, so an increase in occurrence is expected due to climate change (Miraglia *et al.* 2009; Paterson & Lima 2010, 2011). This contamination may potentially cause harm to the fish and shrimps, dependent upon mycotoxin concentration

and co-occurrence, consequently resulting in significant economic losses, directly (e.g. mortality or decreases in performance), or indirectly (e.g. higher susceptibility to diseases). However, one of the biggest barriers to quantify the impact of mycotoxin contamination in the aquaculture industry is the apparent lack of clinical signs or biomarkers in aquatic species for mycotoxin exposure, especially compared to terrestrial livestock. While several reports describe broad and non-specific clinical signs for the most common mycotoxins (see review from Anater *et al.* (2016)), these lack specificity and could be attributed to a number of pathologies or challenges such as the presence of anti-nutrition factors or lectins in the diet (Hart *et al.* 2010). The case of aflatoxicosis, (yellowing of the body surface, Deng *et al.* 2010) and ingestion of fumonisins (FUM; alteration of the sphinganine to sphingosine ratio, Nguyen *et al.* 2003) are two notable exceptions. Also, Gonçalves *et al.* (2018d) described DON-3-sulphate as a potential biomarker of deoxynivalenol (DON) exposure in rainbow trout (*Oncorhynchus mykiss*).

Carry-over denotes the conveyance of undesired compounds from contaminated feed into food of animal origin. The potential of carry-over of several mycotoxins in terrestrial animals such as poultry, swine and cows issue was highlighted by the European Food Safety Authorities (EFSA) and FAO (Domenico Caruso *et al.* 2013; EFSA 2004b FAO 2001). However, no guidelines are available regarding carry-over in farmed fish and shrimp species.

Therefore, the present review aims to compare the mycotoxin occurrence and co-occurrence in aquaculture finished feeds with the potential risk of mycotoxin carry-over in aquaculture seafood products across main aquaculture produced species. Furthermore, we aim to critically compare carry-over obtain in aquaculture species to the ones obtained for livestock species. With this review, we intend to raise awareness to the scientific community, the regulatory authorities and the aquaculture industry to the possible need for specific aquaculture mycotoxin maximum concentration levels for both aquaculture feeds and foods. Furthermore, authors aware for particular cases in aquaculture sector, where edible tissues may change in different regions, therefore increasing the risk of mycotoxicosis.

Occurrence of mycotoxins in aquafeeds

The high cost and limited availability of fishmeal has led the aquaculture industry to gradually increase the levels of alternative protein sources as a substitute for fishmeal in their feeds (Davis & Sookying 2009). Overall, a wide range of products, e.g. animal by-products, fishery by-products, insect meals, macro-algae meals or single-cell protein, have been explored as alternatives to fishmeal. However, for several reasons (e.g. production scalability, market availability, batch uniformity or price competitiveness) plant-based meals remain the most widely used alternative protein source. When considering plant-based meals for aquafeeds, it is commonly agreed that one of the negative aspects is the presence of anti-nutrients (e.g. cyanogens, saponins, tannins, etc.) which are detrimental to fish and shrimp (Krogdahl *et al.* 2010). Conversely, the negative impact of mycotoxins is often overlooked. The disbelief in the negative effects of mycotoxins on aquatic species might be related to the lack of observable clinical signs in aquatic species directly related to mycotoxin ingestion compared to terrestrial livestock species where the effects are more pronounced. However, the awareness of mycotoxin-related issues in the aquaculture industry has grown in recent years as feed manufacturers and producers have recognized the importance of mycotoxins and their potential to impact production, final product quality (García-Morales *et al.* 2013) and safety for consumers (Michelin *et al.* 2017). The evolution of the analytical platforms used to detect mycotoxins and the easier access to analytical labs or simple ELISA strip tests kits for *in situ* testing, has also increased the awareness of mycotoxins to feed millers and farmers.

During the revision of the peer-reviewed literature on the occurrence of mycotoxins in aquafeeds, summarized in this review, a pattern of the target mycotoxins analysed in feed samples emerged. In samples analysed before 2012, the main mycotoxins analysed were AFs (AFB_1, AFB_2, AFG_1, AFG_2; in most of the cases only AFB_1; see Table 1) and in some cases zeralenone (ZEN) and OTA (Fegan & Spring 2007) (with the exception of (Martins *et al.* 2008) and, possibly based on previous data reported on terrestrial livestock feed samples. After 2012, other mycotoxins were beginning to be reported besides AF's (Table 1). These studies have either targeted the analysis of specific mycotoxins due to the inclusion of certain plant meals (e.g. Pietsch *et al.* 2013; Woźny *et al.* 2013) or explored a broad mycotoxin occurrence (Nácher-Mestre *et al.* 2015; Gonçalves *et al.* 2017, 2018c). This different pattern in the target mycotoxin analysed in feed might be a reflection of increasing awareness of mycotoxins in aquaculture, but also as a result of the easier access to mycotoxin analytical methods.

Aquafeed studies with samples preceding 2012

The oldest documented survey of mycotoxin occurrence in aquaculture finished feed was by Bautista *et al.* (1994). In this study, a total of 62 samples collected in the Philippines between August 1990 and February 1991 from black tiger shrimp (*Penaeus monodon*) feed, sourced from feed mills and at farm level were analysis (Table 1). The authors observed that only two of the 62 samples were free from AFs, 36 samples were contaminated with AFs at levels

Table 1 Documented mycotoxin occurrence in aquaculture feeds

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Bautista *et al.* (1994)	August 1990 - February 1991 (rainy season)	Philippines	Feed plant Farm level	n = 62	Black tiger shrimp	AFB_1	n = 2 -> none detected n = 36 -> 10 to 20 μg kg^{-1} AFB_1 n = 21 -> 30 to 40 μg kg^{-1} AFB_1 n = 2 -> 60 to 120 μg kg^{-1} AFB_1	HPTLC	—
Bintvihok *et al.* (2003)	[S]Summer (March - June 1997) [R]Rainy (July - October 1997) [W]Winter (November - February 1998)	Thailand (Eastern and Southern regions)	Farm level	N_t = 150 (50 samples from 10 different regions during 3 seasons)	Black tiger shrimp	AFB_1 AFB_2 AFG_1 AFG_2	Eastern region [S]0.003–0.012 [R]0.003–0.651 [W]0.003–0.314 Southern region [S]0.004 [R]0.003–0.058 [W]0.003–0.022	HPLC	Feeds composed mainly of fishmeal, soybean and corn (no information on ingredient inclusion levels or finished feed storage period)
Altuğ *et al.* (2001)	1998, 1999, 2000	Turkey	Farm level Feed plant Imported feeds	n = 170	Rainbow trout Seabream Pike-perch	AFB_1	n = 20 > 20 μg kg^{-1} n = 85 = 21.2 to 42.4 μg kg^{-1} n = 22 = 5.0 to 20.0 μg kg^{-1} n = 43 < LOD	TLC ELISA	Level of aflatoxins were higher in samples that were taken from farm level compared to feed plant or imported feed samples
Alinezhad *et al.* (2011)	March - July 2009 (1 sample per month)	Iran	Feed plant	n = 6	Rainbow trout	AFB_1	0.12 to 20.09 μg kg^{-1} AFB_1	HPLC	High concentrations of AFB_1 in fishmeal ($\bar{x}$ = 67.35 μg kg^{-1}) and soybean meal ($\bar{x}$ = 30.88 μg kg^{-1})
Fegan and Spring (2007)	n/a	[IN]India [TH]Thailand	n/a	[IN,S]n = 10 [TH,S]n = 7 [TH,F]n = 9	Shrimp[S] Fish[F]	[IN,S](AF, T-2, ZEN) [TH,S](T-2, ZEN, OTA) [TH,F](T-2, ZEN, OTA)	[IN,S]AF = 40–90; (9/10) [IN,S]T-2 = 20–40; (4/10) [IN,S]ZEN = 20–40; (4/10) [TH,S]T-2 = 2.6–50.03; (3/7) [TH,S]ZEN = 16.78–23.00; (6/7) [TH,S]OTA = 2.32–7.74; (7/7) [TH,F]T-2 = 15.91–49.13; (9/9) [TH,F]ZEN = 36.20–118.48; (9/9) [TH,F]OTA = 2.16–9.72; (9/9)	n/a	Marine ingredients (fishmeal from China, Myanmar, Thailand; fish and shrimp meal from Thailand) contaminated with T-2, ZEN and OTA

Table 1 (continued)

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Gonçalves-nunes *et al.* (2015)	January - March 2009	Brazil (Piauí State)	Feed plant	*n* = 18	Fish	AFB_1	1.6–9.8	ELISA	Finished feed samples were composed of soybean bran (15%), corn bran (27%), other cereals (57.5%).
Barbosa *et al.* (2013)	September 2009 and August 2010	Brasil (Rio de Janeiro State)		*n* = 60	n/a	FB_1 AFB_1 OTA	FB_1 = (90%) 0.3–4.94; $\bar{x}$ = 2.6 AFB_1 = present in 55% of the samples OTA = present in 3.3% of the samples No levels mentioned for AFB_1 and OTA	FB_1 - ELISA AFB_1 and OTA - TLC	LOD: 0.2 μg g^{-1} for ELISA (FB_1) 0.003 and 0.005 μg g^{-1} for TLC (AFB_1 and OTA) 50% of samples had co-occurrence of AFB_1 and FB_1 3.3% of the samples tested positive for the three mycotoxins analysed
Martins *et al.* (2008)	n/a	Portugal	n/a	*n* = 20	Fish	AFB_1 OTA DON ZEN FB_1	N.d	HPLC	LOD AFB_1 = 0.2 μg kg^{-1} OTA = 20 μg kg^{-1} DON = 100 μg kg^{-1} ZEN = 50 μg kg^{-1} FUM = 20 μg kg^{-1}

Table 1 (continued)

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Almeida *et al.* (2011)	n/a	Portugal	Feed plant	n = 87	Seabass	AFB_1	AFB_1 n.d. (detection limit of the method was 1.0 μg kg^{-1})	HPLC	35 samples contaminated with *Aspergillus* spp.
Pietsch *et al.* (2013)	n/a	Central Europe	n/a	n = 11	Carp	DON ZEN	DON = 66–825; $\bar{x}$ = 236.18 ZEN = 3–511; $\bar{x}$ = 63.82	HPLC	Most common plant ingredients in feeds collected: C = corn; CGF = Corn gluten feed; SEM = soybean extraction meal; SM = soybean meal; SFEM = sunflower seed extraction meal; W = wheat; WB = wheat bran, WDB = wheat distillery by-product; WGF = wheat gluten feed.
Woźny *et al.* (2013)	November 2012	Poland (North-eastern region)	Farm level	n = 3	Trout	ZEN	$\#_1$ = n.d. $\#_2$ = 81.8 ± 25.8 $\#_3$ = 10.3 ± 0.9	HPLC	Rainbow trout organs were also sampled, refer to table 6.
Greco *et al.* (2015)	n/a	Argentina (Río Negro and Neuquén)	Farm level	n = 28	Rainbow trout	AF OTA T-2 FUM DON ZEN	AF = 1.3–8.91; $\bar{x}$ = 2.82 OTA = 3.5–5.0 $\bar{x}$ = 5.26 T-2 = 50–105.99; $\bar{x}$ = 70.08 FUM = 190–222; $\bar{x}$ = – DON = 150–210; $\bar{x}$ = 230 ZEN = 20.04–159.76; $\bar{x}$ = 87.97	ELISA	Finished feed samples were composed of soybean expeller, disabled soybean, corn, wheat, wheat bran, corn gluten meal Co-occurrence of at least two out of six mycotoxins was recorded in 93% (26/28) of samples analysed

Table 1 (continued)

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Nacher-Mestre *et al.* (2015)	n/a	United Kingdom	Feed plant	n = 5 2 diets^{GSB} with low level plant meal 3 diets^{AS} with high level plant meal	^{AS}Atlantic salmon ^{GSB}Gilthead sea bream	AFB_1, AFB_2, AFG_1, AFG_2, OTA, NEO, FB_1, FB_2, FB_3, T-2, DIA, ZEN, NIV, DON, 3-AcDON, 15-AcDON, FUX, and HT-2	DON^{GSB} = 79.2 and 53.5 DON^{AS} = 22.4, 19.4 and 23.1 FUM^{GSB} = -, 6.4 FUM^{AS} = 148, 754 and 112	LC–MS/MS	No carry-over effects observed after 8^{GSB} and 7AS months of feeding the contaminated diets. Diets manufactured with contaminated ingredients (wheat (n = 3, Germany and Denmark), wheat gluten (n = 4, UK, Germany, and China), pea (n = 1, Denmark), pea protein (n = 2, Norway), rapeseed meal (n = 1, Denmark), corn gluten (n = 3, China and Germany), soya protein (n = 4, Brazil) and sunflower meal (n = 1, Russia).
Gonçalves *et al.* (2018c)	January 2014 – December 2014	^{A}Asia (CN, IN, TH, MN) ^{E}Europe (CR, PT)	Farm level Feed plant	N_t = 41 samples n = 31 Asia n = 6–10 Europe	Shrimp Fish	AF ZEN DON FUM OTA	^{A}AF: $\bar{x}$ = 51.83; Max = 220.61; (21/31) ^{A}ZEN: $\bar{x}$ = 60.41; Max = 232.88; (18/31) ^{A}DON: $\bar{x}$ = 160.86; Max = 413.08; (21/31) ^{A}FUM: $\bar{x}$ = 172.63; Max = 573.32; (18/31) ^{A}OTA: $\bar{x}$ = 2.11; Max = 5.05; (17/31) ^{E}AF: $\bar{x}$ = 0.43; Max = 0.43; (1/6) ^{E}ZEN: $\bar{x}$ = 118.01; Max = 305.89; (4/6) ^{E}DON: $\bar{x}$= 165.61; Max = 281.72 (4/6) ^{E}FUM: $\bar{x}$ = 3419.92; Max = 7533.61; (3/10) ^{E}OTA: $\bar{x}$ = 1.53; Max = 3.1; (4/6)	HPLC	In Europe, 50% of the samples had more than 1 mycotoxin per sample In Asia, 84% of the samples had more than 1 toxin per feed

Table 1 (continued)

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Gonçalves *et al.* (2017)	January – December 2015	[A]Asia (VN, ID, MM) [E]Europe (DK, AT, NL, DE)	Farm level Feed plant	N_t = 25 [A]n = 21 (20/21) [F]n = 4 (4/4)	Shrimp Fish	AF ZEN DON FUM OTA	[A]AF: $\bar{x}$ = 58; Max = 201 [A]ZEN: $\bar{x}$ = 53; Max = 157 [A]DON: $\bar{x}$ = 29; Max = 63 [A]FUM: $\bar{x}$ = 58; Max = 238 [A]OTA: $\bar{x}$ = -; Max = 7 [E]AF: not detected [E]ZEN: $\bar{x}$ = -; Max = 6 [E]DON: $\bar{x}$ = -; Max = 20 [E]FUM: n.d. [E]OTA: n.d.	HPLC	—
Marijani *et al.* (2017)	n/a	Kenya Kisumu -> n = 16 Tanzania Ukerewe -> n = 13 Rwanda Kigembe -> n = 10 Uganda Jinja -> n = 13	[FM]Farm [LFP]Local feed plant [I]Imported feed (from Israel and India) [FI]Feed Ingredients	N_t = 52 [FM]n = 14 [LFP]n = 14 [I]n = 12 [FI]n = 12	Nile tilapia African catfish	3-ADON 15-ADON DON AF DAS AOH FB_1 FB_3 OTA ROQ-C	[FM]AF = 2.4-126; $\bar{x}$ = 71.0 ± 31.5 [FM]FUM = 33.2–2834.6; $\bar{x}$ = 1136.5 ± 717.9 [FM]DON = 69.1–755.4; $\bar{x}$ = 245.8 ± 190.1 [LFM]AF = <2–28; $\bar{x}$ = 11.6 ± 0.7 [LFM]FUM, DON = <LOD [I]AF = <2–2.6; $\bar{x}$ = 1.4 ± 0.9 [I]FUM, DON = <LOD	LC–MS/MS	Farmers who formulate their own feed used: sunflower seed cake, rice bran, cotton seed cake, maize bran and soybean. Feeds co-contaminated with 12[FM], 4[LFM] and 5[I] mycotoxins. NEO, FUX and STERIG were not detected in any of the samples AF co-occurred with FUM in 13 of 24 feed samples DON co-occurred with FUM in 2 of 24 feed samples

Table 1 (continued)

Reference	Sampling year(s)	Sampling Country	Sampling local	Number of samples	Species to which the feed is intended	Target mycotoxin analysed in feed	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Gonçalves *et al.* (2018c)	January – December 2016	Asia (SAS: IN, ID, MN, TW, TH)	Farm level Feed plant	N_t = 16 ^{S}n = 4 ^{F}n = 12	Shrimp^{S} Fish^{F}	AF ZEN DON FUM OTA NIV 3-AcDON 15-AcDON FUX T-2 HT-2 DAS NEO	^{F}AF: $\bar{x}$ = 51.83; Max = 32; (8/12) ^{F}ZEN: $\bar{x}$ = 75.66; Max = 153; (6/12) ^{F}DON: $\bar{x}$ = 82.87; Max = 396; (8/12) ^{F}FUM: $\bar{x}$ = 354.22; Max = 993; (9/12) ^{F}OTA: $\bar{x}$ = 1.65; Max = 3; (6/12) ^{S}AF: $\bar{x}$ = 0.43; Max = 24; (4/4) ^{S}ZEN: $\bar{x}$ = 22.0; Max = 53; (3/4) ^{S}DON: $\bar{x}$ = 881.66; Max = 2287 (3/4) ^{S}FUM: $\bar{x}$ = -; Max = 43; (1/4) ^{S}OTA: $\bar{x}$ = 2.66; Max = 4; (3/4)	LC-MS/MS	——

Reference entries are in chronological ordered by sampling date collection or publishing date. Superscript letters give extra information; they are only valid for the same row.

General abbreviations: $\bar{x}$, average value; $\tilde{x}$, median value; Max, maximum; HPLC, High-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; LC–MS/MS, Liquid chromatography-tandem mass spectrometry; TLC, Thin layer chromatography; HPTLC, high performance thin layer chromatography; LOD, limit of detection; n.d., not detected.

Mycotoxins: AF, aflatoxins (the sum of AFB_1, AFB_2, AFG_1 and AFG_2); AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; DON, deoxynivalenol; FUM, fumonisins (the sum of FB_1 and FB_2); FB_1, fumonisin B_1; FB_2, fumonisin B_2; OTA, ochratoxin A; ZEA, zearalenone; NIV, Nivalenol; 3-AcDON, 3-Acetyldeoxynivalenol; 15-AcDON, 15-Acetyldeoxynivalenol; FUX, fusarenon X-glucoside; fumonisins; DAS, Diacetoxyscirpenol; NEO, neosolaniol; AOH, alternariol; ROQ-C, roquefortine C; STERIG, sterigmatocystin.

Regions: NAS, northern Asia; SAS, South-East Asia; CN, China; IN, India; TH, Thailand; MN, Myanmar; ID, Indonesia; TW, Taiwan; HR, Croatia; PT, Portugal; DK, Denmark; AT, Austria; NL, the Netherlands; DE, Germany.

between 10 and 20 μg kg^{-1}, 21 samples contained AFs at levels between 30 and 40 μg kg^{-1} and two samples had AFs levels of 60 and 120 μg kg^{-1}. The second study was from Bintvihok *et al.* (2003) which analysed samples collected in the eastern and southern regions of Thailand (1997 to 1998) and by Altuğ and Berklevik (2001) with samples collected in Turkey from 1998 to 2000 (Table 1). Bintvihok *et al.* (2003) analysed 150 samples of commercial shrimp feed (formulated for *P. monodon*) composed mainly of fishmeal, soybean and corn. Samples were collected directly from farms in ten different provinces during the summer months (March to June 1997), the rainy season (July to October 1997) and the winter (November to February 1998) and analysed for AFB_1, AFB_2, AFG_1 and AFG_2. Bintvihok *et al.* (2003) observed that feed was more frequently contaminated in the eastern region (43 contaminated out of 75 collected samples) compared to the southern region (14 contaminated out of 75 collected samples). Contamination also occurred more frequently during rainy season (29 contaminated out of 50 collected samples) followed by winter (20 contaminated in 50 collected samples). AFB_1 was the most prevalent mycotoxin found in samples, although at relatively low concentrations (<1 μg kg^{-1}; Table 1). However, the study lacked information regarding levels of inclusion of the plant ingredients as well as storage time and conditions prior to analysis, which does not allow drawing further conclusions regarding the origin of the AF contamination (i.e. from raw materials or contamination during storage). Soybean and corn are not typically contaminated with AFs, at least in the field, as these plant commodities are more likely contaminated with DON, FUM and ZEN (Gonçalves *et al.* 2018a). Therefore, AF contamination in finished feeds could reflect inadequate storage conditions of raw materials or feeds. Reporting inclusion levels of plant ingredients would be very useful. Importantly, Altuğ and Berklevik (2001) analysed 170 fish finished feed samples for the presence of AFB_1 in Turkey between 1998 and 2000. Samples were collected at farm level, feed mills or imported feeds. In this study, AFB_1 was found below detection limits in 43 samples (25.2% of samples), in 20 samples (11.7% of samples) AFB_1 levels were above 20 μg kg^{-1} and in 85 samples (50% of samples) AFB_1 ranged between 21.2 and 42.4 μg kg^{-1}. Authors from this study concluded that levels of AFB_1 were higher in samples taken from farms compared to feed mill or imported feed samples.

Fegan and Spring (2007) reported, to our knowledge, the first and most complete mycotoxin occurrence survey on fish and shrimp feeds before 2012. Samples were collected in India and Thailand and analysed for the presence of AFs, T-2, ZEN and OTA. No information is available on the period of sampling, region area or sample origin (feed mill or farm). Nonetheless, the information reported shows a different contamination pattern between fish and shrimp feeds and also shows co-occurrence of mycotoxins. Out of the nine fish feed samples analysed from Thailand, all samples were contamination predominantly by ZEN, at levels ranging from 36.20 to 118.48 μg kg^{-1}, followed by T-2 (2.6 to 50.03 μg kg^{-1}) and OTA (2.32 to 7.74 μg kg^{-1}). Also in Thailand, shrimp feed samples (n = 7) were contaminated with ZEN and OTA, whereas no data on AFs were available (Table 1). Shrimp feed samples (n = 10) collected from India were mostly contaminated with AFs, ranging between 40 and 90 μg kg^{-1}. However, it is important to mention that levels of sensitivity are mycotoxin-specific and therefore although OTA reported levels were in general lower than ZEN, aquatic species are more sensitive to OTA (see Gonçalves *et al.* 2018c for sensitivity levels in aquatic species). In their study, Fegan and Spring (2007) also reported mycotoxin occurrence in the raw materials used to formulate aquafeeds. While the objective of this review is only to report mycotoxin occurrence in finished feed, it is inevitable and fundamental to highlight the occurrence of mycotoxins (T-2 and ZEN and OTA) in marine ingredients (fishmeal from China, Myanmar, Thailand; fish and shrimp meal from Thailand) which will be further discussed in next sections.

An exception to the almost exclusive AF analysis in finished feeds prior to 2012, are the results presented by Martins *et al.* (2008), who analysed 20 samples of fish feed sourced from Portugal for the presence of AFB_1, OTA, DON, ZEN and fumonisin B1 (FB_1). In this study, no detectable levels of the target mycotoxins were obtained.

In the remaining studies shown in Table 1, in which samples were collected in or before 2012 (Alinezhad *et al.* 2011; Almeida *et al.* 2011; Gonçalves-Nunes *et al.* 2015), the target mycotoxin analysed in feed was always AFB_1. Almeida *et al.* (2011), did not detect AFB_1 in the 87 samples of seabass feed collected in Portugal. Interestingly, 35 of the 87 samples analysed were contaminated with *Aspergillus* spp., which highlights that the presence of fungi does not necessarily mean the presence of the toxin and vice-versa. Alinezhad *et al.* (2011), detected levels high concentrations of AFB_1 in fishmeal (average = 67.35 μg kg^{-1}). In Brasil, Gonçalves-Nunes *et al.* (2015), reported the presence of AFB_1 ranging from 1.6 to 9.8 μg kg^{-1} in samples collected directly at the feed plant.

Aquafeed samples after 2012

From 2012 onwards, the number of peer-reviewed publications and technical articles (not covered in this review) related to the presence of mycotoxins (including not only AFBs) in aquaculture feeds increased considerably. In 2013, Woźny *et al.* (2013) analysed the presence of ZEN in trout feed collected from three farms in November. One of the

farms had no detected levels of ZEN, whereas the other two farms had 81.8 ± 25.8 and 10.3 ± 0.9 μg kg^{-1} of ZEN in their feed respectively. The same study also explored the carry-over of ZEN from feed by analysing several rainbow trout (*O. mykiss*) organs for ZEN presence, results that are further explored in next section. Pietsch *et al.* (2013), unveiled the presence of DON (236.18 μg kg^{-1}) and ZEN (63.82 μg kg^{-1}) in common carp (*Cyprinus carpio*) feeds in samples from central Europe. Still in Europe, Nácher-Mestre *et al.* (2015), investigated the occurrence of mycotoxins in Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*) feeds, with, respectively, high and low inclusion of plant meals. From the 18 mycotoxins analysed, the most representative mycotoxins found were FUM and DON. In Atlantic salmon, from the three types of feeds analysed, levels of DON were 22.4, 19.4 and 23.1 μg kg^{-1} and 148, 754 and 112 μg kg^{-1} of FUM respectively. For gilthead sea bream, two samples were found to contain 79.2 and 53.5 μg kg^{-1} of DON, and 6.4 μg kg^{-1} of FUM in only one of the samples. In Argentina, Greco *et al.* (2015) also analysed salmonids feeds. In this study, 28 samples of rainbow trout (*O. mykiss*) feed were sampled at the farms, ranging throughout the feed portfolio for different development stages (starter feed (13 samples); grower feed (13 samples); four pigmented and nine unpigmented feed and finisher feed (2 pigmented samples)). The authors observed median values of: AFs = 2.82; OTA = 5.26; T-2 = 70.08; DON = 230 and ZEN = 87.97 μg kg^{-1}. It was also highlighted that, there was a co-occurrence of at least two out of six mycotoxins in 93% (26/28) of the analysed samples. Gonçalves *et al.* (2017, 2018a,c) focused on unveiling the mycotoxin occurrence in plant meals (not reported here) and aquaculture finished feeds in Europe and Southeast Asia. In 2014, from January to December, 41 fish and shrimp feed samples were collected from Europe (n = 6 to 10; Croatia and Portugal) and SE Asia (n = 31; Singapore, India, Thailand and Myanmar). Samples were analysed for AFs, ZEN, DON, FUM and OTA (Table 1). Interestingly, a higher occurrence of FUM was found in European samples (average 3,419.92 and maximum 7,533.61 μg kg^{-1}) compared to SE Asia. The remaining mycotoxins showed similar occurrence average and maximum levels for Europe and SE Asia, with mycotoxins being detected in all analysed samples. In this mycotoxin survey (Gonçalves *et al.* 2018c), it was reported that in Europe, 50% of the samples had more than one mycotoxin per sample, and in Asia, 84% of the samples were contaminated with more than one mycotoxin per feed sample.

In 2015, analysing the same mycotoxins as in the previous study, Gonçalves *et al.* (2017) sourced 25 samples of fish and shrimp feeds in Europe (n = 4; Denmark, Austria, Netherlands and Germany) and SE Asia (n = 21; Vietnam, Indonesia, Myanmar). Contrary to samples collected in 2014, the European samples analysed in 2015 showed relatively low mycotoxin contamination, with only DON contamination reaching values up to 20 μg kg^{-1}. In SE Asian samples, contamination was also generally lower when compared to the previous year, with only AFs showing similar contamination levels to 2014 (average contamination of 58 μg kg^{-1} and maximum of 201 μg kg^{-1}). However, the co-occurrence risk increased in both regions.

From January to December 2016, Gonçalves *et al.* (2018a) sampled four shrimp feeds from India and 12 fish feeds from Indonesia, Myanmar, Taiwan and Thailand. Interestingly, the fish and shrimp feeds showed a relatively different mycotoxin contamination pattern, possibly due to the type of raw materials used to manufacture these diets. Fish feed samples showed lower contamination (Table 1), when compared with shrimp feeds. However, a higher number of co-occurring mycotoxins were observed in fish feeds. Shrimp feeds showed a relatively high contamination of DON, with an average contamination level of 881.66 and maximum of 2,287 μg kg^{-1}.

Mycotoxins also represent a big challenge to the increasingly successful aquaculture sector on the African continent. Marijani *et al.* (2017), analysed mycotoxin occurrence in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) feeds, gathering 16 samples from Kisumu, Kenya, 13 samples from Ukerewe, Tanzania, 10 samples from Kigembe, Rwanda and 13 samples from Jinja, Uganda. Samples were collected from farms (farm-made feeds; n = 14), local feed millers (n = 14) or imported feeds from Israel and India (n = 12). From the 52 samples analysed, Marijani *et al.* (2017) observed that farm-made feeds were highly contaminated with AF, FUM and DON (Table 1). On the other hand, feed samples from local feed millers, as well as the imported feed samples, had only minor contamination of AF.

Discussion on the occurrence of mycotoxins in aquafeeds

From the documented peer-reviewed literature, it is possible to observe a growing interest in the occurrence of mycotoxins in aquatic feeds. It is also observable that there is a shift regarding the target mycotoxins analysed in feeds. Most of the earlier studies evaluating mycotoxins in aquafeeds (Altuğ & Berklevik 2001; Bintvihok *et al.* 2003) mainly focused on aflatoxin occurrence and only in recent years, other mycotoxins were analysed. This research pattern, that is high focus on AFs and only later on other mycotoxins, can also be observed in the peer-reviewed literature studying the impact of mycotoxins in aquatic animal health and performance (Gonçalves *et al.* 2018c). The increasing interest in mycotoxins in aquafeeds, and particularly the interest in other mycotoxins besides AFs, is certainly related to the increasing inclusion levels of plant

meals in aquafeeds, as well as, the awareness of mycotoxins conveyed from these plant meals to aquafeeds. However, we cannot exclude the easier access to analytical instrumentation to determine mycotoxins together with the evolution of the analytical methods *per se* as a plausible contribution to this shift.

The results of the most recent mycotoxin occurrence surveys of aquaculture feeds (Nácher-Mestre *et al.* 2015; Gonçalves *et al.* 2017, 2018a, 2018c; Marijani *et al.* 2017) clearly show an increase in mycotoxin occurrence compared to previous surveys (Altuğ & Berklevik 2001; Bintvihok *et al.* 2003; Alinezhad *et al.* 2011; Almeida *et al.* 2011). Unfortunately, it cannot be concluded, from this data, that there is a higher mycotoxin risk now compared to the past. This is because the target mycotoxins analysed in older studies were not the same and sensitivity detection levels and methodologies have since improved significantly. Nonetheless, it was theoretically expected that an increasing level of plant meals in aquafeeds would lead to increased occurrence of mycotoxins in these feeds, which is observable by the most recent occurrence surveys (Nácher-Mestre *et al.* 2015; Gonçalves *et al.* 2017, 2018c; Marijani *et al.* 2017).

Besides the increasing mycotoxin occurrence and the focus on a broad range of mycotoxins, several other important conclusions can be taken from the studies summarized in Table 1. A key aspect is the regional differences in mycotoxin occurrence reported and the correlation between fungi contamination and the presence of mycotoxins. The presence of moulds in a fish feed is the first indication that something is wrong with its hygiene. There are several reasons why feeds get mouldy, from improper storage conditions (high humidity, high variations in temperatures leading to condensation, etc.) to poor manufacturing process (e.g. insufficient drying time, lack of preservatives/anti-moulds, etc.). Fungi contamination can also originate from inappropriate selection of ingredients, which can carry fungi spores that are resistant to extrusion/pelleting, having the capacity to germinate afterwards (due to improper storage or poor manufacturing processes).

While the presence of fungi might be a direct risk for the host, for example *Fusarium oxysporum* and *Fusarium solani*, known as opportunistic pathogens for fish and shrimp (Hatai *et al.* 1986; Ostland *et al.* 1987; Lightner 1996; Souheil *et al.* 1999), and an indirect risk which reduces the palatability and therefore intake of the feed, its presence does not necessarily correlate with the presence of the toxin producer mould and *vice-versa* (Alinezhad *et al.* 2011; Greco *et al.* 2015). On the other hand, mycotoxins produced on crops in the field will remain in raw materials, even after processing, due to their heat stability (Pitt 2014), whereas fungi will be destroyed due to high temperatures. For example *Fusarium* spp. are field fungi usually lacking the ability to grow on dry feed. However, the toxins produced by these fungi species (e.g. DON, FUM) will remain stable on the plant raw materials used to manufacture aquafeeds, and in some cases, even be redistributed and concentrated in certain milling fractions (Cheli *et al.* 2013), for example corn *vs* corn gluten meal (Gonçalves *et al.* 2018a). Mycotoxin redistribution and transfer from crops to aquafeeds has been observed and reported by Gonçalves *et al.* (2018a). While it is not the core of the present review, we need to highlight that, with the exception of AF and OTA, most of the other mycotoxins found in the occurrence surveys and shown in Table 1 are probably due to the use of plant meals rather than mycotoxins being produced during storage. So, the selection and analysis of the plant raw materials selected to manufacture aquafeeds is the first step to minimize mycotoxin accumulation risks in aquafeeds.

The regional differences in mycotoxin occurrence are also an important factor which cannot be overlooked. Fungal growth, and consequently mycotoxin production in crops, is influenced by several factors, with weather conditions being the most important (Miraglia *et al.* 2009; Paterson & Lima 2010, 2011). Consequently, it could be expected that different regions present differences in mycotoxin contamination patterns, and even within a region, mycotoxin occurrence may vary depending on seasonal conditions. This is shown by the data reported by Bintvihok *et al.* (2003) in samples from Thailand, which suggests that rainy seasons might be more problematic and therefore should be closely monitored. However, factors such as climate change and the world trade of commodities makes it challenging to estimate the risk of mycotoxins in aquaculture finished feeds. For example as reported by Gonçalves *et al.* (2018c), higher levels of FUM in European finished feeds compared to SE Asia samples cannot be easily explained and therefore a better understanding on the origin of sourced ingredients is necessary. The increasing globalization of trade commodities and incorporation of imported raw materials into aquafeeds exposes the industry to the potential risk of mycotoxins, which are sometimes not even common for the region (not the case in that particular study). Therefore, mycotoxin contamination needs to take into account the globalization of raw materials, which could already have significant levels of mycotoxins together with the monitoring of finished feeds.

Emerging mycotoxins

Emerging mycotoxins are a class of mycotoxins which its occurrence in feed and food commodities has been increasing only recently (Kovalsky *et al.* 2016) and which may represent a potential toxicity towards animals and humans. The presence of these mycotoxins also produced

by *Fusarium* spp. (as are DON, FUM and ZEN described previously) is expected to increase due to climate change (Miraglia *et al.* 2009; Paterson & Lima 2010, 2011). However, quantitative estimates of their occurrence are scarce, especially in aquaculture feeds. While for trichothecenes, data on its toxicity, occurrence and contamination levels are available, reported in previous section, for other metabolites also produced by *Fusarium* spp., such as moniliformin (MON), fusaproliferin (FUS), beauvericin (BEA) or enniatins (ENNs), limited information is available. Moreover, the typical *Fusarium* mycotoxins (DON, FUM and ZEN) are legislated for certain levels in feed commodities, however, for this new diverse group of 'emerging toxins', for example MON, FUS, BEA and ENNs, legislation is scarce (Kovalsky *et al.* 2016). Besides that, the effects of these mycotoxins on aquaculture species is still relatively unknown (Yildirim *et al.* 2000; Nguyen *et al.* 2003, 2003; Jestoi 2008; Gonçalves *et al.* 2018c). Generally, is observed that, regulated mycotoxins, that is FUM, DON and ZEN occurrence levels in feeds are still higher than these emerging mycotoxins (Kovalsky *et al.* 2016). However, Tolosa *et al.* (2013) identified several enniatins (ENNs; ENA1, ENB and ENB1) in seabream, seabass, tilapia and panga tissues from commercialized aquaculture fish. To our knowledge, Tolosa *et al.* (2013) study is the first of its kind and highlights for the need to better understand mycotoxin carry-over beyond the typical *Fusarium* spp. mycotoxins. This topic will be further discussed in section 'Data obtained from commercially sourced aquaculture products'.

Carry-over of mycotoxins

Bioaccumulation of mycotoxins from feed to animal food products might represent a direct risk to human health (CAST 2003). Mycotoxin bioaccumulation in livestock is well investigated (I. Völkel *et al.* 2011; Leeman *et al.* 2007) and the risk to humans is currently being evaluated by the European Food Safety Authority (EFSA) for several mycotoxins (AF, OTA, ZEN, DON, FUM, T-2 and HT-2). Bioaccumulation of mycotoxins in poultry, swine and cows is managed by direct regulation of mycotoxins in animal feed (EFSA 2004a,c,d, 2005, 2011, 2013, EC 2006). While regulatory limits have been put in place for AFs (), only guidance values are available for DON, OTA, FUM and zearalenone (ZEN; EC 2006). This is because feed does not represent a direct risk for human health and because carry-over of these mycotoxins in terrestrial animals is expected to be low (EC 2006).

Currently, no regulations or guidelines exist in order to avoid deposition of mycotoxins in farmed fish or shrimp, with the exception of fumonisins (FB1 + FB2 = 10 mg kg^{-1}; EC 2006). Moreover, it is not taken into consideration that carry-over mechanisms in aquaculture farmed species might be different from terrestrial livestock species. Generally, the possibility of mycotoxin bioaccumulation/biomagnification through the food chain due to the use of mycotoxin contaminated non-plant origin ingredients such as animal by-products (e.g. shrimp head meal or chicken droppings (further discussed in section 'Carry-over data obtained from feeding trials'; 'Aflatoxins')) or non-typical mycotoxin contaminated ingredients (e.g. fishmeal), is not taken into consideration and will be addressed during this review.

Bioaccumulation of mycotoxins in aquaculture seafood products is not widely reported and consequently not regulated. This section will focus on documented peer-reviewed mycotoxin carry-over studies focussed in aquaculture species. Existing literature is reviewed, calculating transfer factors when the available data allow it, in order to compare bioaccumulation risks (Leeman *et al.* 2007). The transfer factor is expressed as the concentration of mycotoxin in animal tissues (μg kg^{-1}) divided by the concentration of the same mycotoxin in animal feed (μg kg^{-1}).

Carry-over data obtained from feeding trials

The present section intends to give an overview of studies reporting the carry-over of mycotoxins from feed to animal tissues, assessed in feeding trials with supplemented mycotoxins in feed. We calculated transfer factors for carry-over of mycotoxins from feed to eggs, whole milk, meat and edible offal as calculated by Leeman *et al.* (2007) (Table S1). The data presented by Leeman *et al.* (2007) covered 250 references resulting in a comparison of 3,624 transfer factors from livestock species (cattle, poultry, pig, sheep, goat, rabbit, pheasant, turkey, duck and quail). These authors took into account the carry-over of AFs (AFB_1, AFB_2, AFG_1 and AFG_2), DON, OTA, T-2 and ZEN. Leeman *et al.* (2007) reported average transfer factors, ignoring the differences in different mycotoxin kinetics as well as the different metabolism capacity of animals. Nonetheless, the information gathered has a high relevance and allows a first comparison between transfer factors in aquaculture-farmed species *vs* livestock.

Aflatoxins (AFs)

Aflatoxin bioaccumulation from feed to animal tissues is well documented for aquaculture species. A total of 19 studies have evaluated the presence of AFs in fish and crustacean tissues after being fed a certain amount of this same mycotoxin (Table 2).

The first study (Suzy *et al.* 2017) reported in Table 2 raises an interesting and not yet discussed point about the occurrence of mycotoxins in feed conveyed from animal by-products and not necessarily from plant meals. Suzy

et al. (2017) reported that with increasing aquaculture production in Africa, in this case the West Cameroon region, feed ingredients are a serious limitation to the sustainable growth of the aquaculture sector. The author reported that due to the good protein content, chicken droppings were being used as an ingredient in the local fish food or as direct feed, despite its contamination with AF's. Suzy *et al.* (2017) reported that after feeding African sharptooth catfish (*C. gariepinus*) with 10, 17 and 20 μg AFB_1 kg^{-1}, for three months, 0.05 ± 0.12, 0.08 ± 0.10 and 0.08 ± 0.12 μg AFB_1 kg^{-1} of AFB_1 were found in muscle tissue samples respectively. Calculated transfer factors (0.004–0.005) (Table 2) for AF in the muscle are within range to values reported for eggs and meat (Leeman *et al.* 2007).

Regarding cold/temperate water reared species, five studies are available; in European seabass (*Dicentrarchus labrax*) (El-Sayed & Khalil 2009), hybrid sturgeon (*Acipenser ruthenus* × *A. baeri*) (Rajeev Raghavan *et al.* 2011), walleye fish (*Sander vitreus*) (Hussain *et al.* 1993) and rainbow trout (*O. mykiss*) (Ngethe *et al.* 1992, 1993; Ellis *et al.* 2000) (Table 2). Studies in rainbow trout so far have used tritium (^3H) to label AFB_1 and it has been not possible to obtain the amount (in μg kg^{-1}) of AFB_1 in tissues. Both authors detected AFB_1 in several samples (faeces, kidney, gastro-intestinal tract, carcass, urine and bile (Ellis *et al.* 2000); bile, liver, kidney, brain, abdominal fat, muscle, spleen and blood (Ngethe *et al.* 1992); liver and brain (Ngethe *et al.* 1993)) up to six (Ngethe *et al.* 1993), seven (Ellis *et al.* 2000) and eight (Ngethe *et al.* 1992) days after ingestion of AF. El-Sayed and Khalil (2009), after feeding seabass with 18 μg kg^{-1} of AFB_1, detected 4.25 ± 0.85 μg AFB_1 kg^{-1} in muscle samples, which correspond to a TF of 0.278, which is higher than that observed for livestock meat (Table S1). Reported values in muscle in this study (4.25 ± 0.85 μg AFB_1 kg^{-1}) are considerably high if one considers that the regulatory limit for AFB_1 in human foods set by the US Food and Drug administration is 5 μg kg^{-1}. Also, in walleye fish (*S. vitreus*), Hussain *et al.* (1993) reported high levels of AFB_1, AFB_2, AFG_1 and AFG_2 in muscle, which generated TF of 0.1 to 0.5, which are comparable to what is obtained for edible offal and higher than that observed for livestock meat (Table S1). In the case of the Hybrid sturgeon (*A. ruthenus* × *A. baeri*), animals fed with 40 μg AF kg^{-1} feed, showed values of 28 μg kg^{-1} of AF in muscle and 142.80 μg kg^{-1} in the liver (TF = 0.7 and 3.57) (Rajeev Raghavan *et al.* 2011), whereas when fed with 80 μg kg^{-1} AF the TF were lower both in muscle and liver (TF = 0.4 and 1.15).

Tropical species have been particularly studied covering both Asian and South American species. Regarding Nile tilapia (*O. niloticus*) eight studies have been published to date (Salem *et al.* 2009; Deng *et al.* 2010; Ayyat *et al.* 2013; Hessein *et al.* 2014; Selim *et al.* 2014; Mahfouz & Sherif 2015; Abdel Rahman *et al.* 2017; Hussain *et al.* 2017). All studies detected bioaccumulation of AF in muscle and the liver (Table 2). However, these studies vary in terms of fed mycotoxin levels as well as tilapia development stages. Mahfouz and Sherif (2015), used tilapias with an initial weight of 35 ± 0.50 g, and fed them with 20 or 100 μg kg^{-1} AF for 12 weeks, with intermediary sampling at six weeks (Table 2). This study found that both AF levels led to accumulation in the liver and muscle, however, in the liver, AFs were found earlier (six weeks post-intake) than in the muscle (only after 12 weeks). The intake period is an important factor to take into consideration as shown by Mahfouz and Sherif (2015), and equally important would be to establish suitable depuration periods for the different mycotoxins. If feasible, adequate fasting periods before harvesting which currently vary from species to species could be set according to mycotoxin tissue levels. Despite using a considerably high range of AFB_1 levels in his study, Deng *et al.* (2010) observed during a 20 week trial, that even relatively low AFB_1 levels (85 μg kg^{-1}) could lead to a significantly high accumulation of AFB_1 in the liver after 20 weeks of ingestion (AFB_1 in the liver after 20 weeks = 30 μg kg^{-1}; Table 2). In short exposure periods to AF (30 days), Abdel Rahman *et al.* (2017) observed that the intake of 200 μg kg^{-1} of AF accumulated in the liver and muscle at 5 ± 0.5 and 3.7 ± 0.1 μg kg^{-1} respectively. This might suggest a certain incapability to metabolize AF.

Other studies also performed in tilapia (*O. niloticus*) (Salem *et al.* 2009; Ayyat *et al.* 2013; Selim *et al.* 2014), support the previously reported studies, but show a tendency for a higher accumulation of AFs in muscle (Table 2), which could be related to the smaller size of the tilapias used (7 to 15 grams). For example Selim *et al.* (2014) reported the deposition of 90 μg kg^{-1} of AFs in the muscle after feeding tilapia (15 ± 2 g) with 200 μg kg^{-1} of AF for 10 weeks. Likewise, the Ayyat *et al.* (2013) and Salem *et al.* (2009) studies that used fish with an initial weight of 7.3 g and 10 g, respectively, also showed high values of AFs in the muscle (78.33 μg kg^{-1} and 99.48 μg kg^{-1} respectively). In comparison, in the study by Mahfouz and Sherif (2015) that used fish with an initial weight of 35 g, intake of 100 μg kg^{-1} AF over 12 weeks led to a lower accumulation of AF in the muscle (0.05 μg kg^{-1}). This tendency for higher AF deposition in younger animals seems to be further confirmed by Hessein *et al.* (2014), where after feeding tilapias of 7.3 grams for 98 days with 250 μg kg^{-1} AF, an AF deposition of 101.7 μg kg^{-1} was found. This means a TF of 0.407 that, together with data reported by previous authors (Salem *et al.* 2009; Selim *et al.* 2014), have relatively high TFs for muscle and are only comparable to livestock edible offal (Table S1).

Finally, Hussain *et al.* (2017) showed a high deposition of AF in tilapia muscle, however, the levels of mycotoxins

Table 2 Documented aflatoxin carry-over on aquaculture species

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Fish studies						
Suzy *et al.* (2017)	**African sharptooth catfish** (*Clarias Gariepinus*)	10^1, 17^2 and 20^3 μg AFB_1 kg^{-1}	M^1 = 0.05±0.12 μg AFB_1 kg^{-1} M^2 = 0.08 ± 0.10 μg AFB_1 kg^{-1} M^3 = 0.08 ± 0.12 μg AFB_1 kg^{-1}	M^1 = 0.005 M^2 = 0.005 M^3 = 0.004	ELISA	Initial weight: 4 ± 2 g; 3 month study Chicken droppings were used as ingredient contaminated with 5, 7.2 and 8.2 μg AFB_1 kg^{-1} Catfish fed 10 μg AFB_1 kg^{-1} used as control No differences in haematological parameters
El-Sayed and Khalil (2009)	**European seabass** (Dicentrarchus labrax)	#1Oral 96 h LC_{50} > 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 mg kg^{-1} #242 day exposure to 10% of oral 96 h LC_{50} = 180 μg kg^{-1}	#2M = 4.25 ± 0.85 μg AFB_1 kg^{-1}	#2M = 0.236	ELISA	Initial weight: 40 ± 2 g #196 h LC_{50} = 0.18 mg/kg bwt #20.018 mg/kg bwt AFB_1 #1,2 Clinical signs: sluggish movement, loss of equilibrium, rapid opercular movement, and haemorrhages of the dorsal skin surface. #2Yellowish discoloration, pale discoloration of the gills, liver and kidney. Severe distension of the gall bladder.
Huang *et al.* (2011)	**Gibel carp** (*Carassius gibelio*)	3.2, 11.3, 20.2^1, 55.2^2,95.8^3, 176.0^4 and 991.5^5 μg AFB_1 kg^{-1}	$L^{1\text{-}5}$ > 5 μg AFB_1 kg^{-1} M^5 = 2.35 μg AFB_1 kg^{-1}	$HP^{1\text{-}5}$* > 0.090 M^5 = 0.0024	ELISA	Initial weight: 10.33 ± 0.19 g 12 week study Fish showed strong clearance ability of AFB_1
Rajeev Raghavan *et al.* (2011)	**Hybrid sturgeon** (*Acipenser ruthenus* × *A. baeri*)	0, 1, 5, 10, 20, 40^1 and 80^2 μg AFB_1 kg^{-1}	M ≈ 28^1 and 34^2 L = 142.80^1 and 115.60^2 μg kg^{-1}	M^1 = 0.7 M^2 = 0.425 L^1 = 3.57 L^2 = 1.4453.4	ELISA	Initial weight: 10.53 ± 0.17 g 35 day study Liver hypertrophy and hyperchromasia of nuclei and cytoplasmic vacuoles, presence of inflammatory cells, focal hepatocyte necrosis and extensive biliary hyperplasia.
Lopes *et al.* (2009)	**Jundiá** (*Rhamdia quelen*)	#1 41, 90^1 and 204^2 μg AFB_1 kg^{-1} #2$350^1$; 757^2; $1{,}177^3$ μg AFB_1 kg^{-1}	#1M = 1^1 and 6.1^2 μg AFB_1 kg^{-1} #2M + L = 3.4^1; 7.1^2 μg kg^{-1} and 19.6^3 μg AFB_1 kg^{-1}	#1M^1 = 0.024 #1M^2 = 0.030 #2M + L^1 = 0.010 #2M + L^2 = 0.009 #2M + L^3 = 0.017	HPLC	Initial weight: 3.21#1 g and 4.73#2 g 45#1 and 35#2 day studies

Table 2 (continued)

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Michelin *et al.* (2017)	**Lambari fish** (*Astyanax altiparanae*)	0, 10^1, 20^2 and 50^3 μg AFB_1 kg^{-1}	L = $265^{2,1}$ and $243^{3,1}$ μg kg^{-1} M = $19^{1,1}$, $20^{2,1}$ and $50^{3,1}$ μg kg^{-1}	$L^{2,1}$ = 13.25 $L^{3,1}$ = 4.86 $M^{1,1}$ = 1.9 $M^{2,1}$ = 1 $M^{3,1}$ = 1	HPLC	Initial weight: 3.15 g 120 day study (sampling at day 30, 60, 90 and 120^1) For the first 60 days of exposure, AFs were metabolized by liver and excreted. After 90 days, a lower efficiency in the elimination of AFs
Abdel Rahman *et al.* (2017)	**Nile tilapia** (*Oreochromis niloticus*)	(0 and 200^1 μg AFB_1 kg^{-1}) × (FEO + SC)	L^1 = 5 ± 0.5 μg AFB_1 kg^{-1} M^1 = 3.7 ± 0.1 μg AFB_1 kg^{-1}	L^1 = 0.025 M^1 = 0.019	HPLC	Initial weight: 26.6 ± 0.12 g; 30 day study Tested fennel essential oil (FEO) and saccharomyces cerevisiae (SC) as mycotoxin management strategy. AF effects are reported only for 0 and 200^1 μg kg^{-1}
Ayyat *et al.* (2013)	**Nile tilapia** (*Oreochromis niloticus*)	(0, 250^1 μgAFB_1 kg $feed^{-1}$) × OZ, B or C	M^1 = 78.33 μg kg^{-1}	M^1 = 0.313	HPLC	Initial weight: 7.3 g; 3 week study Tested ozone (0.5 mg/L/minute; OZ), bentonite (20 g/kg diet; B) and coumarin (5 g/kg diet; C) as detoxifying strategy
Deng *et al.* (2010)	**Nile tilapia** (*Oreochromis niloticus*)	19; 85^0; 245^1; 638^2; 793^3 and $1{,}641^4$ μg kg^{-1}	$Y^{t1\text{-}tf,0\text{-}4}$ L^{t1} = 10^0, 16^1, 21^2, 24^3 and 24^4 μg AFB_1 kg^{-1} liver L^{tf} = 30^0, 33^1, 47^2, 44^3 and 43^4 μg AFB_1 kg^{-1} liver	$Y^{t1\text{-}tf,0\text{-}4}$ L^{t1} = 0.118^0, 0.065^1, 0.033^2, 0.030^3 and 0.015^4 L^{tf} = 0.353^0, 0.135^1, 0.074^2, 0.055^3 and 0.026^4	ELISA	Initial weight: 20 g; 20^{tf} week study (sampling at week 5^{t1}) AF from mouldy peanut meal
Hessein *et al.* (2014)	**Nile tilapia** (*Oreochromis niloticus*)	(0, 250^1 mg kg^{-1}) × Vit or C	M^1 = mg kg^{-1}	M^1 = 0.407	HPLC	Initial weight: 7.3 g; 98 day study Tested coumarin (5 g/kg diet; C) and vitamin E (50 mg kg^{-1} diet; Vit) as detoxifying strategy No differences on Hb, RBcs, Hct, WBCs, Plat Note: Hessein *et al.* 2014 reports in his manuscript a residual AF of 107.7 mg kg^{-1}, each seems extremely high, which might be a mistake of units mg kg^{-1}/μg kg^{-1}

Table 2 (continued)

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Hussain *et al.* (2017)	**Nile tilapia** (*Oreochromis niloticus*)	(0, 2000[1], 4000[2] mg kg^{-1}) × 0.5% and 1% CB	M^2 = 0.087 ± 1.32 μg kg^{-1}	M^2 ~ 0	HPLC	Initial weight: 4.5 ± 0.4 g; 10 week study Tested calcium bentonite (CB) clay as detoxifying strategy; Tested CB significantly improved some parameters (WG, HIS) CB significantly reduced bioaccumulation of AFB_1 residues in muscle tissues.
Mahfouz and Sherif (2015)	**Nile tilapia** (*Oreochromis niloticus*)	20[1] and 100[2] μg AFB_1 kg^{-1} feed	$L^{1,t1}$ = 5 μg kg^{-1} $L^{1,t2}$ = 8 μg kg^{-1} $L^{2,t1}$ = 10 μg kg^{-1} $L^{2,t2}$ = 15 μg kg^{-1} $M^{2,\ t2}$ = 5 μg kg^{-1}	$L^{1,t1}$ = 0.25 $L^{1,t2}$ = 0.4 $L^{2,t1}$ = 0.1 $L^{2,t2}$ = 0.15 $M^{2,\ t2}$ = 0.05	TLC	Initial weight: 35 ± 0.50 g; 6^{t1} or 12^{t2} week studies Challenge test with *Aeromonas hydrophila*, *iP.* Expression of liver *GPx* and *GST* down-regulated[1] The ability to withstand *A. hydrophila* infection was remarkably lowered
Salem *et al.* (2009)	**Nile tilapia** (*Oreochromis niloticus*)	0, 150[1] μg AFB_1 kg^{-1}	M^1 = 99.48 μg AFB_1 kg^{-1}	M^1 = 0.663	HPLC	Initial weight: 10 ± 3 g; 15 week study AFB_1 was produced through pellet fermentation using *Aspergillus parasiticus* NRRL 2999
Selim *et al.* (2014)	**Nile tilapia** (*Oreochromis niloticus*)	(0 and 200[1] μg kg^{-1}) × HSCAS, SC and EGM	M^1 ≈ 90 μg kg^{-1}	M^1 ≈ 0.45	HPLC	Initial weight: 15 ± 2 g; 10 week study Tested hydrated sodium calcium aluminosilicates (HSCAS; 0.5%), *Saccharomyces cerevisiae* (S.C.; 0.25%) and an esterified glucomannan (EGM; 0.25%) as detoxifying strategy; AF produced from polished raw rice
Ngethe *et al.* (1993)	**Rainbow trout** (*Oncorhynchus mykiss*)	15.6 μg mL^{-1} of AFB_1	$L^{1,\ 2,\ 4}$ $B^{1,\ 2,\ 4}$	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	Initial weight: 200 ± 20 g; 3 week study (sampling at 6 h^1, 1 day^2, 2 $days^3$ and 6 $days^4$) Intravenous injection of 3H-AFB_1

Table 2 (continued)

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Ellis *et al.* (2000)	**Rainbow trout** (*Oncorhynchus mykiss*)	20 μg kg^{-1} AFB1 and 20 μg kg^{-1} AFB_1 + 2% clay	Detected in: F, K, Gl, U, Bi, Ca	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	Initial weight: 266 ± 12.6 g, 7 day study 2% sodium bentonite Volclay tested as detoxifying strategy;
Ngethe *et al.* (1992)	**Rainbow trout** (*Oncorhynchus mykiss*)	15.6 μg mL^{-1} of AFB_1	Detected in: Bi, L, K, B, AbF, M, Sp and Bl	n/a	[^{3}H]-AFB_1 was measured in a scintillation counter and data expressed in counts per minute (CPM)	Initial weight: 100 ± 15 g, 8 day study (sampling at 6 h, 1, 2 4 and 8 days) Intravenous injection and oral dose of ^{3}H-AFB_1
Usanno *et al.* (2005)	**Red tilapia** (*Oreochromis niloticus* × *O. massambicus*)	0, 50, 100, 500, 1,000 and 2,500 μg kg^{-1}	Not detected	n/a	n/a	8 week trial No information on fish weight
Hussain *et al.* (1993)	**Walleye fish** (*Sander vitreus*)	0, 50 and 100[1] μg kg^{-1}	Detected in muscle: AFB_1[1] = 5 μg kg^{-1} AFB_2[1] = 10 μg kg^{-1} AFG_1[1] = 15 μg kg^{-1} AFG_2[1] = 20 μg kg^{-1}	AFB_1 = 0.5 AFB_2 = 0.1 AFG_1 = 0.15 AFG_2 = 0.2	n/a	30 day study No information on fish weight
Shrimp studies						
Boonyaratpalin *et al.* (2001)	**Black tiger shrimp** (*Penaeus monodon Fabricius*)	0; 50[1]; 100[2]; 500[3]; 1,000[4]; 2,500[5] μg kg^{-1} AFB_1	Head and shell/muscle (μg kg^{-1}) [1,11] = 2.6/13.0; [1,12] = 0.5/0.4 [2,11] = 3.5/14.2; [2,12] = -/0.6 [3,11] = 9.1/10.6; [3,12] = 6.8/0.3 [4,11] = 2.3/8.4; [4,12] = 6.5/0.7 [5,11] = 3.9/7.4; [5,12] = 4.9/0.1	Head and shell/muscle (μg kg^{-1}) [1,11] = 0.052/0.26; [1,12] = 0.01/0.008 [2,11] = 0.035/0.142; [2,12] = -/0.006 [3,11] = 0.0182/0.0212; [3,12] = 0.0136/0.0006 [4,11] = 0.0023/0.0084; [4,12] = 0.0065/0.0007 [5,11] = 0.0016/0.0030; [5,12] = 0.0020/–0	TLC	Study in adult stage, Initial weight: 1.0-1.2 g; 8 week trial (sampling at 4[11] and 6[12] weeks)
Bintvihok *et al.* (2003)	**Black tiger shrimp** (Penaeus monodon Fabricius)	5, 10, 20 μg kg^{-1} AFB_1	not detected	n/a	HPLC	Study in adult stage 10 day trial AFB_1 was prepared from mouldy corn

Table 2 (continued)

Reference	Species	Tested dosage	Mycotoxin detection level (µg kg^{-1})	Transfer factor	Method of analysis	Observations
Bautista *et al.* (1994)	**Black tiger shrimp** (Penaeus monodon Fabricius)	25, 50, 75, 100 or 200 µg kg^{-1} AFB_1	Not detected	n/a	HPTLC	Study in adult stage, initial weight: 17.5 ± 0.6 g 62 day trial

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding the mycotoxin contamination, when not mentioned it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC, High-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; TLC, Thin layer chromatography; LOD, limit of detection; nd, not detected; n/a, not applicable.

Tissue abbreviations: M, Muscle; L, Liver; HP, hepatopancreas; B, Brain; F, faeces; K, Kidney; GI, Gastro intestinal tract; U, Urine; Bi, Bile; Ca, carcass; AbF, abdominal fat; Sp, spleen and Bl, blood.

used in this trial (2,000–4,000 µg kg^{-1}) are unlikely to be found in aquafeeds although TFs calculated for AF deposition in the liver are in line with the other studies. The only trial with red tilapia (*O. niloticus* × *O. mossambicus*), (Usanno *et al.* 2005) reported no detectable levels of AF in tilapia tissues, after being fed AF levels ranging from 50 to 2,500 µg kg^{-1}.

The deposition of AFs in the liver and muscle of Gibel carp (*Carassius gibelio*) are similar to the levels reported for Nile tilapia (Huang *et al.* 2011).

Lopes *et al.* (2009) reported the deposition of AFs in the liver and muscle in Jundiá (*Rhamdia quelen*) fed low (41.90 and 204 µg kg^{-1}) and high (350, 757 and 1,177 µg kg^{-1}) AF levels for 45 and 35 days respectively. Focusing on lower AF levels, as they are within the observed AF's occurrence levels in aquafeeds, 41.90 µg AF kg^{-1} feed led to the deposition of 1 µg kg^{-1} in the muscle and 204 µg kg^{-1} of AFs led to the deposition of 6.1 µg kg^{-1} AFs. These bio-accumulation level of AFs leads to TFs of 0.02, which is comparable to the level of accumulation on livestock edible offal's ((Leeman *et al.* 2007); Table S1).

Lambari fish (*Astyanax altiparanae*), a native central/south American small fish (10–15 cm length and 60 g), has been seen as a potential aquaculture species for rural population in Brasil. Michelin *et al.* (2017) reported lambari fish as highly prone to AF deposition in the liver and muscle. After lambari fish were fed 20 kg^{-1} of AFs for 120 days, deposition of AFs in the liver was 265 µg kg^{-1} (TF 13.5) and in fish fed 50 µg kg^{-1} AFs levels in the liver were 243 µg kg^{-1} (TF 4.86). This level of bio-accumulation in the liver is higher than the bioaccumulation of highly liposoluble mycotoxins in terrestrial animal fat (Leeman *et al.* 2007; Table S1). Such AFs levels in this species could be particularly challenging as these fish are normally eaten as snacks, that is the entire fish is deep-fried, dried and/or salted.

Reports of AF carry-over in shrimp are limited to three studies performed in black tiger shrimp (*P. monodon*). Two of these studies (Bautista *et al.* 1994; Bintvihok *et al.* 2003) did not find any AF residues after feeding shrimps with different AF concentrations (5 to 200 µg kg^{-1}) for 10 and 62 days respectively. In contrast, Boonyaratpalin *et al.* (2001) found AF residues in cephalothorax and in muscle, after feeding the shrimps AFB_1 levels ranging from 50 to 2,500 µg kg^{-1} with TF values ranging from 0.006 to 0.052. Contextualizing the AF contamination levels found in feed around SE Asia (<500 µg kg^{-1}; Fegan & Spring 2007; Gonçalves *et al.* 2018a,c, 2017) with the Boonyaratpalin *et al.* (2001) study, shrimps fed AFB_1 levels of 50 and 100 µg kg^{-1} led to considerably high AF deposition in head and shell (2.6 and 3.5 µg kg^{-1} AFB_1 respectively) and in muscle (13 and 14.2 µg kg^{-1} AFB_1 respectively), after 4 weeks of AFB_1 intake. For the same intake amounts (50

and 100 μg kg^{-1} AFB_1), AFB_1 deposition levels in head/shell and muscle samples decreased over time (after 6 weeks; Table 2). This might suggest a certain capacity to eliminate or metabolize AFB_1.

Ochratoxins (OTA)

Ochratoxin bioaccumulation studies in aquaculture-farmed species are very scarce. The most comprehensive study was carried out by Bernhoft *et al.* (2017) in Atlantic salmon (*S. salar*). Bernhoft *et al.* (2017) studied the deposition of OTA in liver, muscle, kidney and skin samples after feeding salmon with 800 or 2,400 μg kg^{-1} of OTA for 8 weeks. Deposition of OTA in kidney and skin samples was not detected (except in kidney for high intake dosage after 8 weeks, Table 3). In muscle samples, OTA levels were under the limit of quantification. Major deposition was observed in the liver, however, a bioaccumulation over the exposure period was not found, with the highest OTA deposition peaking after 3 weeks (both for ingestion of 800 and 2,400 μg kg^{-1} OTA). This suggests that Atlantic salmon might have the ability to eliminate OTA. Previously, OTA deposition in salmonids (rainbow trout (*O. mykiss*)) was investigated by Fuchs *et al.* (1986) where the deposition of OTA in several organs (Table 3) was analysed up to 8 weeks after an intravenous injection of OTA (0.160 μg kg^{-1}). Authors observed that OTA deposition in the kidney and bile was persistent during the whole trial, also suggesting the action of the kidney in detoxification mechanism of OTA. The only study reporting carry-over of OTA in shrimp (*P. monodon*) was by Supamattaya *et al.* (2005a), which did not detect OTA deposition in tissues after feeding shrimps with OTA levels ranging from 100 to 1,000 μg kg^{-1}. However, the limit of detection given in the manuscript (44,000 μg kg^{-1}) seems to be particularly high for HPLC, suggesting a possible error in the units reported.

Deoxynivalenol (DON) and fumonisins (FUM)

Deoxynivalenol and/or FUM bioaccumulation data in aquaculture species is summarized in Table 4. Similar to OTA, DON and FUM carry-over effects in aquaculture-farmed are scarce. In Atlantic salmon (*S. salar*), two studies are available (Nácher-Mestre *et al.* 2015 and Bernhoft *et al.* 2017). Bernhoft *et al.* (2017) fed salmon with 2,000 and 6,000 μg kg^{-1} DON over the course of 8 weeks and sampling liver, muscle, kidney and skin at 3, 6 and 8 weeks. The authors observed that both exposure dosages (2,000 and 6,000 μg kg^{-1} DON) led to DON deposition in the liver and muscle at all sampling points, except for the higher dosage at the last sampling point (8 weeks), at which DON was found in all sampled tissues (Table 4). In the case of the study performed by Nácher-Mestre *et al.* (2015), Atlantic salmon were fed lower levels of mycotoxins, however, with multi-occurrence. The three diets were mainly formulated with DON and FUM, but also minor levels of T-2 and 15-AcDON (Table 4). Salmon fed for 6 months with testing diets did not show detectable levels of DON and FUM in the tissues studied. The same authors (Nácher-Mestre *et al.* 2015) also studied bioaccumulation of mycotoxin co-occurrence (DON, 15-AcDON and FUM) in Gilthead sea bream (*S. aurata*) at two levels for 8 months. The authors did not observe mycotoxin deposition in muscle samples.

In common carp (*C. carpio*), Pietsch *et al.* (2014) observed that after feeding fish with 352, 619 and 953 μg kg^{-1} DON for 4 weeks, minor deposition of DON was observed in the muscle (Table 4). Interestingly, after the 4 weeks of DON exposure, fish were fed a non-contaminated diet for a period of 2 weeks and DON levels in the muscle were re-analysed. At the lower DON intake level (352 μg kg^{-1}), DON level in the muscle was higher after the depuration period (1.4 μg kg^{-1}) when compared with the level found at the end of feeding trial (8 weeks; 0.6 μg kg^{-1} DON). At the medium DON intake level (619 μg kg^{-1}), after the recovery period, a level of 0.7 μg kg^{-1} DON was still found in the muscle, and at the higher level, however, no DON was detected after the recovery period.

In shrimps, two studies are available (Trigo-Stockli *et al.* 2000; Supamattaya *et al.* 2005b; Table 4), in which both reported that DON was not detected in the muscle. Supamattaya *et al.* (2005b) drew its conclusion after feeding black tiger shrimp black (*P. monodon*) with 500, 1,000 and 2,000 μg kg^{-1} DON for 8 weeks. Trigo-Stockli *et al.* (2000) conducted its study using Pacific white shrimp (*Litopenaeus vannamei*), fed with 200, 500 and 1,000 μg kg^{-1} DON for 16 weeks.

Zeralenone (ZEN)

Zearalenone (ZEN) is a regular contaminant of cereal crops worldwide, and being a phytoestrogenic compound (Diekman & Green 1992), is mainly responsible for oestrogenic agonist-related effects (Marasas 1991). As a hormone mimicking substance, ZEN can bind to oestrogen receptors in target cells (Kumar *et al.* 2013). Generally, ZEN studies have focused mainly on dysfunction or structural disorders in the reproductive tract of farm animals (Zinedine *et al.* 2007; Minervini & Aquila 2008; Woźny *et al.* 2013). While it seems that ZEN does not directly affect the growth performance of aquaculture-farmed species, its deposition in fish tissues seems to be common and already well documented particularly in cold water species (Arukwe *et al.* 1999; Pietsch *et al.* 2015; Woźny *et al.* 2015, 2017).

In common Carp (*C. carpio*), Pietsch *et al.* (2015) found that after exposing fish to 4 weeks with 332, 621 and 797 μg ZEN kg^{-1} feed, minor residues of ZEN and α-ZEN

Table 3 Documented ochratoxin carry-over in aquaculture species

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Fish studies						
Bernhoft *et al.* (2017)	**Atlantic Salmon** (*Salmo salar*)	0, 800[1] and 2,400[2] μg kg^{-1} OTA	L/M/K/SK (μg kg^{-1}) [1,t1] = 1.86/<LOQ/n.s./n.s. [1,t2] = 1.53/<LOQ/n.s./n.s. [1,t3] = 1.01/<LOQ/0.16/n.s. [2,t1] = 4.81/<LOQ/n.s./n.s. [2,t2] = 3.27/<LOQ/n.s./n.s. [2,t3] = 2.61/<LOQ/1.03/n.s.	L/M/K/SK [1,t1] = 0.0023/<LOQ/n.s./n.s. [1,t2] = 0.0020/<LOQ/n.s./n.s. [1,t3] = 0.0012/<LOQ/~0/n.s. [2,t1] = 0.0020/<LOQ/n.s./n.s. [2,t2] = 0.0013/<LOQ/n.s./n.s. [2,t3] = 0.0011/<LOQ/~0/n.s.	HPLC	Initial weight: 58 g Administration of 14C-OTA A and autoradiography Sampling at 3[t1], 6[t2] and 8[t3] weeks
Fuchs *et al.* (1986)	**Rainbow trout** (*Salmo gairdneri*)	IV injection of 0.160 μg kg^{-1}	Blood = Detected[t1-t4] Pronephros = Detected[t1-t4] Opisthonephros = Detected[t1-t4] Urine = Detected[t1-t4] Pseudobranch = Detected[t1-t4] Gills = Detected[t1-t4] Liver = Detected[t1-t4] Bile = Detected[t1-t4] Ventricle wall = Detected[t1-t4] Pyloric appendices = (contents) = Detected[t1-t4] Large intestine (contents) = Detected[t1-t4] Spleen ('patches') = Detected[t1-t4] Muscle (close to the myomeres) = Detected[t1-t2] Spinal cord = Detected[t1-t3] Fins = Detected[t1-t4] Skin = Detected[t1-t4] Muscles = Detected[t1-t2]	n/a	LC fluorometer	Initial weight: 50 g, 8 week study Sampling at 5 min[t1], 6[t2] and 8[t3] weeks Fish each was sacrificed at 5[t1] min, 1[t2] h, 24[t3] h and 8[t4] days after injection
Shrimp studies						
Supamattaya *et al.* (2005a,b)	**Black tiger shrimp black** (*Penaeus monodon Fabricius*)	100; 200 and 1,000 μg kg^{-1}	Not detected	n/a	HPLC	Initial weight: 2 g; 8 week study No differences on THC or Ca^{2+} levels No differences in tissues: G, AG, HP, HT, * LOD given in the manuscript (44,000 μg kg^{-1}) seems to be very high; there is a chance of an error in the units

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC, High-performance liquid chromatography; LC, liquid chromatography; n/a, not applicable; n.s. not sampled.

Tissue abbreviations: M, Muscle; L, Liver; K, Kidney; SK, skin.

Table 4 Documented deoxynivalenol and/or fumonisin carry-over in aquaculture species

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Fish studies						
Bernhoft *et al.* (2017)	**Atlantic salmon** (*Salmo salar*)	0; 2000[1] and 6000[2] μg kg^{-1} DON	L/M/K/SK (μg kg^{-1}) [1,t1] = 12.2/5.6/n.s./n.s. [1,t2] = 12.8/8.5/n.s./n.s. [1,t3] = 18.1/6.0/12.3/n.s. [2,t1] = 9.6/10.3/n.s./n.s. [2,t2] = 20.2/17.3/n.s./n.s. [2,t3] = 28.6/18.6/16.8/20.8	L/M/K/SK [1,t1] = 0.0061/0.0028/n.s./n.s. [1,t2] = 0.0064/0.0042/n.s./n.s. [1,t3] = 0.0091/0.003/0.0061/n.s. [2,t1] = 0.0016/0.0017/n.s./n.s. [2,t2] = 0.0034/0.0029/n.s./n.s. [2,t3] = 0.0048/0.0031/ 0.0028/0.0035	HPLC	Initial weight: 58 g, 8 week study; Sampling at 3[t1], 6[t2] and 8[t3] weeks
Nácher-Mestre *et al.* (2015)	**Atlantic salmon** (*Salmo salar*)	Diet 1 = 22.4 DON + 148 FUM Diet 2 = 19.4 DON + 754 FUM Diet 3 = 23.1 DON + 112 FUM	Not detected	n/a	LC–ESI–MS/MS	6 month trial Initial body weight of 228 ± 5 g Minor amounts of T-2 found and 15-AcDON and OTA detected
Pietsch *et al.* (2014)	**Common carp** (*Cyprinus carpio*)	352[1], 619[2] and 953[3] μg kg^{-1} DON	Muscle samples (μg kg^{-1}) [1] = 0.6;[1, RP] = 1.4 [2] = 1.3;[2, RP] = 0.7 [3] = 1.2;[3, RP] = 0.0	Muscle samples [1] = 0.0017;[1, RP] = 0.0040 [2] = 0.0021;[1, RP] = 0.0011 [3] = 0.0013;[1, RP] = 0	HPLC	Raised from eggs (average initial weight 36 g), 4 week study Additional 2 weeks of feeding uncontaminated diet – recovery period[RP]
Nácher-Mestre *et al.* (2015)	**Gilthead sea bream** (Sparus aurata)	Diet 1 = 79.2 DON + 8.1 15-AcDON Diet 2 = 53.5 DON + 13.6 15 – AcDON + 6.4 FUM	Not detected	n/a	LC–ESI–MS/MS	8 month trial Initial body weight of 15 g up to 296–320 g
Huang *et al.* (2018)	**Grass carp** (*Ctenopharyngodon idella*)	27; 318[1]; 636[2]; 922[3]; 1,243[4] and 1,515[5] μg kg^{-1} DON	PI = 16.46[4]; 17.64[5] μg kg^{-1} tissue MI = 15.90[3]; 18.54[4]; 20.34[5] μg kg^{-1} tissue DI = 18.91[3]; 24.40[4]; 28.82[5] μg kg^{-1} tissue	PI = 0.013[4]; 0.012[5] MI = 0.017[3]; 0.015[4]; 0.013[5] DI = 0.021[3]; 0.020[4]; 0.019[5]	HPLC	Initial weight: 12.17 ± 0.01 g; 60 days trial Malformations: missing of pelvic fin[2]; caudal fin deformity[3]; operculum 'the safe dose of DON for grass carp were all estimated to be 318 μg/kg diet'; Huang *et al.* 2011;
Shrimp studies						
Supamattaya *et al.* (2005b)	**Black tiger shrimp black** (*Penaeus monodon Fabricius*)	500; 1,000 and 2,000 μg kg^{-1} DON	Not detected	n/a	HPLC	Initial weight: 2 g; 8 week study No differences on THC or Ca^{2+} levels No differences in tissues: G, AG, HP, HT, * LOD given in the manuscript (50,000 μg kg^{-1}) seems to be very high; there is a chance of an error in the units

Table 4 (continued)

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Trigo-Stockli *et al.* (2000)	**Pacific white shrimp** (*Litopenaeus vannamei*)	0, 200, 500 and 1,000 μg kg^{-1} DON	Not detected	n/a	HPLC	Initial weight: 1.7 ± 0.05 g; 16 week study (sampling at 4, 8, 12 and 16 weeks) Naturally contaminated hard red winter wheat
Deng *et al.* (2017)	**Pacific white shrimp** (*Litopenaeus vannamei*)	0; 500[1]; 1,200[2]; 2,400[3]; 4,800[4]; 12,200[5] μg kg^{-1} T-2	^{HP}m = 17.52 ± 2.87[4] ng g^{-1} ^{HP}m = 48.61 ± 3.13[5] ng g^{-1}	n/a	TSQ	Initial weight: 8.5 ± 0.5 g; 20 days study Dietary concentrations correspond to $^1/_{50}$, $^1/_{20}$, $^1/_{10}$, $^1/_5$ and $^1/_2$ (Wang *et al.* 2015)

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC, High-performance liquid chromatography; LC–ESI–MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; TSQ, Quantum Access tandem mass spectrometer n/a, not applicable; n.s. not sampled.

Tissue abbreviations: M, Muscle; L, Liver; K, Kidney; SK, skin.

were found in the muscle. Interestingly, after 2 weeks of depuration, α-ZEN was not detected and ZEN levels in the muscle decreased significantly (Table 5).

Woźny *et al.* (2015, 2017) dedicated significant efforts at understanding the potential of ZEN bioaccumulation in fish, using mainly rainbow trout as a model. The authors found that after feeding rainbow trout with 1,810 μg ZEN kg^{-1} feed for 71 days, ZEN was found at a concentration of 732.2 μg kg^{-1} in the intestine, whereas non-quantifiable levels of ZEN were found in liver and female ovaries. In another trial, Woźny *et al.* (2017) used mature females (1,274 ± 162 g) to study ZEN carry-over into eggs. Authors found that ZEN is transferred from the gastrointestinal tract to the reproductive system of the fish, depositing ZEN metabolites in the somatic cells of the ovaries rather than in the oocytes.

Discussion on the carry-over data obtained from feeding trials

In order to take realistic conclusions regarding the risk of mycotoxin consumption from aquaculture seafood products, it is necessary to have a good overview of mycotoxin occurrence in aquaculture feeds, and to have quality data on mycotoxin bioaccumulation in aquatic species.

From all the studies regarding AF carry-over presented in Table 2, a few of them should be excluded due to the use of high levels of AFs (Hussain *et al.* 2017); or higher dosages, which are not normally observed in commercial feeds (Boonyaratpalin *et al.* 2001; Usanno *et al.* 2005; Deng *et al.* 2010). The studies reported by the remaining authors, employed plausible dietary mycotoxin levels, identifying the carry-over of AFs in several important species.

From these studies, it is possible to conclude that AFs might represent a serious risk for human consumption, especially in cases where fish are eaten as a whole. In general, transfer factors are quite high for these aquaculture species, being comparable with transfer factors for eggs, whole milk and in some cases for edible offal's or fat of livestock provenience.

In the case of European seabass, mycotoxin levels tested by El-Sayed and Khalil (2009) (18 μg kg^{-1}), which is a mycotoxin level very plausible to be obtained in commercial diets led to 4.25 ± 0.85 μg AFB_1 kg^{-1} in the muscle,. As shown by Altuğ and Berklevik (2001) (Table 1), of the 170 samples collected in Turkey, which is the main EU seabass producer, 105 samples were contaminated with AFs at levels higher than 20 μg kg^{-1}. Regarding hybrid sturgeon (*A. ruthenus*), there is no available mycotoxin occurrence data for this species, even in regions where it is predominantly produced. However, in-feed concentrations tested by Rajeev Raghavan *et al.* (2011), which led to the accumulation of AF in the muscle and liver, seem realistic (40 to

Table 5 Documented zearalenone carry-over in aquaculture species

Reference	Species	Tested dosage	Mycotoxin detection level (μg kg^{-1})	Transfer factor	Method of analysis	Observations
Fish studies						
Pietsch *et al.* (2015)	**Common Carp** (*Cyprinus carpio* L.)	0; 332[1]; 621[2] and 797[3] μg kg^{-1}	Muscle ZEN^{1} = 0.13 ± 0.03 μg kg^{-1} ZEN^{2} = 0.22 ± 0.18 μg kg^{-1} ZEN^{3} = 0.15 ± 0.07 μg kg^{-1} α-ZEN^{1} = 0.11 ± 0.03 μg kg^{-1} α-ZEN^{2} = 0.16 ± 0.011 μg kg^{-1} α-ZEN^{3} = 0.05 ± 0.07 μg kg^{-1} ZEN$^{1,\ RP}$ = 0.03 ± 0.03 μg kg^{-1} ZEN$^{2,\ RP}$ = 0.03 ± 0.02 μg kg^{-1} ZEN$^{3,\ RP}$ = 0.03 ± 0.03 μg kg^{-1}	Muscle ZEN^{1} ~ 0 ZEN^{2} ~ 0 ZEN^{3} ~ 0 α-ZEN^{1} ~ 0 α-ZEN^{2} ~ 0 α-ZEN^{3} ~ 0 ZEN$^{1,\ RP}$ ~ 0 ZEN$^{2,\ RP}$ ~ 0 ZEN$^{3,\ RP}$ ~ 0	HPLC	Raised from egg with 12–16 cm in length 4 week study α-ZEN were not detectable after recovery period (2 weeks) and ZEN was detected at 0.03 μg kg^{-1} dry weight for all treatments
Woźny *et al.* (2015)	**Rainbow trout** (*Oncorhynchus mykiss*)	1,810 μg kg^{-1}	Intestines ZEN = 732.2 μg kg^{-1} α-ZEN = 10.7 μg kg^{-1} L = residual ZEN and α-ZEN in all sampled fish	Intestines ZEN = 0.40 α-ZEN = 0.0059	HPLC	Initial weight: 250 g, all females; 71 day study Some animals were identified as males ZEN was detected (<5.0 μg kg^{-1}) in all female ovaries
Woźny *et al.* (2017)	**Rainbow trout** (*Oncorhynchus mykiss*)	1 mg kg^{-1} of body mass	ZEN/α-ZEN/β-ZEN (μg kg^{-1}) I$^{48\ h}$ = ~1,500/~600/- I$^{96\ h}$ = ~1,500/~900/- L$^{48\ h}$ = ~700/~100/~500 L$^{96\ h}$ = <200/<20/~0 O$^{48\ h}$ = 321/~100/- O$^{96\ h}$ = <100/<100/- Oo$^{48\ h}$ = ~25/~10/- Oo$^{96\ h}$ = <5/<5/- P$^{48\ h}$ = ~10/~5/- P$^{96\ h}$ = ~0/~0/- M$^{48\ h}$ = ~5/~5/- M$^{96\ h}$ = ~3/~3/-	ZEN/α-ZEN/β-ZEN (μg kg^{-1}) I$^{48\ h}$ = 1.5/0.6/- I$^{96\ h}$ = 1.5/0.9/- L$^{48\ h}$ = 0.7/0.1/0.5 L$^{96\ h}$ = <0.2/<0.02/~0 O$^{48\ h}$ = 0.321/0.1/- O$^{96\ h}$ = <0.1/<0.1/- Oo$^{48\ h}$ = ~0.025/~0.01/- Oo$^{96\ h}$ = <0.005/<0.005/- P$^{48\ h}$ = ~0.01/~0.005/- P$^{96\ h}$ = ~0/~0/- M$^{48\ h}$ = ~0.005/~0.005/- M$^{96\ h}$ = ~0.003/~0.003/-	HPLC-FLD	Initial weight: 1,274 ± 162 g, all mature females Objective was to study the ZEN carry-over to eggs Administration on ZEN – oral (bolus) Sampling periods: 2, 6, 12, 24, 48, 72, 96 h Verified the presence of ZEN and α-ZEN in commercial fish roe 'Contamination of fish roe with zearalenone residuals is unlikely to pose a health risk to consumers, but their potential to transfer to somatic cells in fish ovaries may be of concern for aquaculture', Woźny *et al.* 2017
Shrimp - no studies						

Reference entries are alphabetically ordered by species common name. Superscript letters give extra information; they are only valid for the same row. Regarding mycotoxin contamination, when not mentioned, it is assumed that a purified form of the respective mycotoxin was used.

General abbreviations: HPLC, High-performance liquid chromatography; HPLC-FLD, High-performance liquid chromatography with fluorescence detection.

Tissue abbreviations: I, Intestines; O, Ovaries; Oo, Oocytes; P, Plasma, M, Muscle.

80 μg AFB1 kg^{-1}) and therefore further research should be carried out to determine mycotoxin levels in feed for this species and AF accumulation in eggs (caviar).

Carry-over effects on Nile tilapia are well described. Taking into account the available occurrence of AF in tilapia producing countries, that is Brasil (Barbosa *et al.* 2013), S/SE Asian countries (Fegan & Spring 2007; Gonçalves *et al.* 2017, 2018a,c) and Africa (Marijani *et al.* 2017) together with bioaccumulation studies, carry-over of AF in Nile tilapia might represent a challenge worth of further investigation. From the previously cited studies, it is also important to highlight that exposure period is an important factor to take into consideration. Chronic exposure to low AF levels (AF = 85 μg kg^{-1} for 20 weeks) could lead to a significantly high accumulation in the liver (AF in the liver after 20 weeks = 30 μg kg^{-1}, Deng *et al.* 2010). However, short exposure periods should not be undervalued, as periods as short as 30 days can lead to considerable AF deposition in the liver and muscle (Abdel Rahman *et al.* 2017).

Aflatoxin carry-over studies in shrimp are more limited than in fish species. Furthermore, the information available is contradictory, as two studies (Bautista *et al.* 1994 and Bintvihok *et al.* 2003) did not find any AF residues in tiger shrimp muscle, whereas Boonyaratpalin *et al.* (2001) found AF bioaccumulation in head/shell and in the muscle. Results suggested a minor bioaccumulation over time (TFs; Table 2), highlighting a certain capacity to eliminate or metabolize AFB_1. However, levels of AF found in the muscle (13 μg kg^{-1} AFB_1) after feeding shrimps 50 μg kg^{-1} of AFB_1 for 4 weeks were considerably high and could be a threat for human food safety. AF deposition, especially in head samples, should not be undervalued. In many countries, heads are used for direct human consumption. Unfortunately, no information is available for Pacific white leg shrimp (*Litopenaeus vannamei*) which is the most important produced shrimp species in terms of volume.

For OTA occurrence, little information is available for aquaculture feeds, however, according to available studies, levels below 10 μg kg^{-1} have been reported (Fegan & Spring 2007; Greco *et al.* 2015; Gonçalves *et al.* 2017, 2018a,c). The risk of OTA carry-over was only successfully addressed in Atlantic salmon and partially in rainbow trout. In Atlantic salmon (Bernhoft *et al.* 2017), it would appears that OTA is rapidly eliminated. Its deposition in tissues was only shown in liver (4.81 μg kg^{-1}) and only at the highest OTA intake level (2,400 μg kg^{-1}). These OTA levels are unlikely to be observed in commercial feeds. In rainbow trout, OTA deposition in the muscle was not detected after 24 h of OTA intake. This again suggests a rapid elimination of OTA and decreases the risk for human consumption as fasting periods before slaughter in salmonids are normally longer than 24 h. However, it is highly recommended that more studies are undertaken on OTA carry-over, especially for species were OTA occurrence in feeds is more frequent and higher, such as tropical species, where fasting periods before harvest also tend to be much shorter than for cold-water species and also tropical crustacean species.

DON, FUM and ZEN occurrence in aquafeeds have been well documented in recent years (Pietsch *et al.* 2013; Greco *et al.* 2015; Nácher-Mestre *et al.* 2015; Gonçalves *et al.* 2017, 2018a,c; Marijani *et al.* 2017). These mycotoxins have been pointed out as the main mycotoxin contaminants in aquaculture feeds, which is a reflection of the increasing inclusion levels of plant meals in diets, as these mycotoxins are produced in field conditions. However, DON and FUM bioaccumulation has been poorly studied in aquaculture-farmed species. In Atlantic salmon, two interesting and complementary studies are available (Nácher-Mestre *et al.* 2015 and Bernhoft *et al.* 2017). While Bernhoft *et al.* (2017) proved the possibility of DON deposition in the liver and muscle in a relatively short exposure period (3 weeks) with high DON levels (2,000 and 6,000 μg kg^{-1} DON), Nácher-Mestre *et al.* (2015) showed no carry over effects of FUM and DON co-contamination at low levels during long exposure periods. DON and FUM frequently occur together in aquaculture feeds as both mycotoxins are produced by the same fungi species. Therefore, studies testing the effect of co-occurrence are particularly relevant. The levels tested were within the occurrence values reported in European aquafeeds (Gonçalves *et al.* 2017, 2018c), however, occasional high occurrences of DON and/or FUM should not be ignored (e.g. FUM occurrence reported by Gonçalves *et al.* (2018c)), as shown previously, levels up to 2,000 μg kg^{-1} can lead to DON deposition in the muscle.

Contrary to Atlantic salmon, in common carp (*C. carpio*), Pietsch *et al.* (2014) showed that levels as low as 352 μg kg^{-1} DON can lead to a minor deposition of DON in the muscle (Table 4). The author described that total DON elimination from the muscle is a relatively long process, taking more than 2 weeks after stopping DON intake. Information about the complete elimination of DON is very important, as a fasting period before harvesting may be used to guarantee that DON or any other mycotoxin is eliminated during this period. However, in the study reported by Pietsch *et al.* (2014), the elimination period of DON in carp may be longer than the fasting period, which is normally 24 to 48 h before harvesting. The study by Pietsch *et al.* (2014) highlighted that mycotoxin absorption, distribution, metabolism and excretion (ADME) is entirely dependent on species, and data or conclusion extrapolations between species should be avoided. *Fusarium* mycotoxins (e.g. DON and FUM) are frequently present in plant commodities used for general aquaculture species, and taking into account the possible ADME

differences depending on species and even on development stages, it would be very important to better understand the potential carry-over in the most important aquaculture species, giving a special emphasis to mycotoxin co-occurrence.

Despite the low number of studies on DON and FUM carry-over, apparently, its deposition in tissues seems to be very limited. However, its occurrence is frequent and due to its apparently long elimination period (generally higher than fasting period before slaughter, for the study species), its carry-over risk in aquaculture-farmed species should be better evaluated. Comparing TFs obtained from Atlantic salmon and common carp, it seems that they are in line with the TFs of eggs, whole milk or meat (Table S1, Leeman *et al.* 2007).

Is also important to highlight that the species investigated so far are cold/temperate water species. It is essential to increase the knowledge on the possible carry-over of *Fusarium spp.* mycotoxins in tropical species. Especially high value species, normally exported, such as Pacific white leg shrimp, whose feeds have been identified recently as being contaminated with considerably high levels of DON (Gonçalves *et al.* 2018a). Furthermore, these tropical species present a faster metabolism and consequently lower fasting period before harvest is need, which might greatly influence the deposition of mycotoxins in tissues.

From the few available studies evaluating ZEN carry-over effects, it is possible to conclude that, at least for the cold-water species studied so far (common carp and rainbow trout), ZEN and its metabolites can be deposited in several tissues, including muscle, intestine, liver, ovaries and oocytes. However, the levels found in these tissues, with the exception of the intestine and liver (Table 5, Woźny *et al.* 2017), are rather low and do not pose a direct risk to human consumption. In the European Union, the maximum allowable level of ZEN ranges from 20 μg kg^{-1} for processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young, to 300 μg kg^{-1} for unprocessed maize (not for human consumption) (EC 2006). However, European legislation does not include limits for the concentration of ZEN residuals in food of animal origin, since it is thought that carry-over of the *Fusarium* mycotoxins (including DON and FUM previously discussed) to meat, milk and eggs is only minimal (EC 2006; CONTAM 2011).

Moreover, ZEN and its metabolites seem to be more easily deposited in the somatic cells of the ovaries rather than in the oocytes. For rainbow trout and common carp, tissues such as ovaries, liver and intestines are not typically edible, however, for other species this might not be the case. It would be very important to assess the carry-over of ZEN and its metabolites for other aquaculture-farmed species, taking into account what is already known in rainbow trout and common carp. It is particularly interesting to evaluate species that reach sexual maturation before or near harvesting size. ZEN in feed may accelerate the sexual maturation of the fish, leading to energy losses to gonad development, and in some cases organoleptic and physical changes of the final product. For some species, ZEN in feed may also have potential implications for fish and shrimp spawning and further studies need to address this topic. In addition, fish/shrimp species that might be consumed entire, that is including tissues such as the liver, intestines and ovaries should be taken into consideration, as ZEN might reach considerably high levels in these tissues. In certain cases, the use of fish/shrimp by-products in direct human consumption (fish oil) or as an ingredient to formulate new products, should also be taken into consideration as *Fusarium* mycotoxins tend to be quite stable to processing conditions and only minor degradation is expected.

Data obtained from commercially sourced aquaculture products

Table 6 documents mycotoxin occurrence in commercially sourced aquaculture products. Evaluating the occurrence of mycotoxins directly in fish/shrimp products from aquaculture provenience obtained from commercial farms or local supermarkets is a good strategy to evaluate the potential risk of mycotoxin carry-over from feeds to fish/shrimp edible products. Tolosa *et al.* (2013) analysed several samples (n = 19) of fish from aquaculture and wild fishery provenience bought locally in Spain. The author analysed samples for the presence of beauvericin (BEA) and enniatins (enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB) and enniatin B1 (ENB1)). As expected, no mycotoxins were detected in the wild fishery samples. ENA and BEA were also not detected in the aquaculture samples. However, ENA1, ENB and ENB1 were detected in most of aquaculture samples (Table 6). Detecting enniatins in aquaculture foods might lead us to two hypothesis. First, that other *Fusarium* mycotoxins (FUM, DON and ZEN mainly) were probably at even higher concentration levels and are not reported as they were not analysed. The second hypothesis is the fact that ENNs might be more easily deposited in the muscle compared to DON/FUM, even if present at lower levels in aquafeeds. As it is known that ENNs normally occur together with the main *Fusarium* mycotoxins (FUM, DON), it would also be important to study if this synergistic presence in the tissues might lead to increased deposition of certain mycotoxins or metabolites. While it is difficult to evaluate the importance of detecting ENNS in aquaculture foods, these results highlight the need to better study the adverse effects of dietary mycotoxins on fish health and welfare, and consequently carry-over risks.

Table 6 Documented mycotoxin occurrence in commercial aquaculture foods

Reference	Sampling Country (region)	# samples/Species	Sample origin	Target mycotoxin analysed in tissue	Tissue sampled	Mycotoxin detection level (μg kg^{-1})	Method of analysis	Observations
Tolosa *et al.* (2013)	Spain (Valencia)	N_t = 19 n = 9 ^{SB}Seabass^{AQ} n = 5 ^{GSB}Seabream^{AQ} n = 3 (mackerel, hake, cod)^{WF} n = 1 ^{T}Tilapia^{AQ} n = 1 ^{P}Panga^{AQ}	Aquaculture^{AQ} Seabass Spain (Cartagena, Murcia) Greece (Argolis) Seabream Spain and Greece (Argolis); Tilapia China Pangasius Vietnam Wild fisheries^{WF} Hake Southeast Atlantic Cod and Mackerel Northwest Atlantic	BEA ENA ENA1 ENB ENB1	Muscle	ENA1^{SB} = 1.70 ± 0.07 to 6.91 ± 0.12; 4/9 n.d. ENA1^{GSB} = 2.48 ± 0.07 to 7.45 ± 0.12; 2/5 n.d. ENA1 = 1.51 ± 0.07^{T}; n.d.^{P} ENB^{SB} = 3.60 ± 0.08 to 44.65 ± 0.12; 1/9 n.d. ENB^{GSB} = 1.30 ± 0.08 to 21.63 ± 0.11; 1/5 n.d. ENB = 5.35 ± 0.07^{T}; 1.26 ± 0.06^{P} ENB1^{SB} = 1.44 ± 0.09 to 31.51 ± 0.11; 2/9 n.d. ENB1^{GSB} = 7.13 ± 0.1 to 18.95 ± 0.12; 2/5 n.d. ENB1 = 2.20 ± 0.07^{T}; n.d.^{P} ENA1/ENB/ENB1^{WF} = nd	LC–MS/MS	ENA and BEA were not detected in samples analysed Seabass (*Dicentrarchus labrax*) Seabream (*Sparus aurata*) Aquaculture^{AQ} Wild fisheries^{WF}
Woźny *et al.* (2013)	Poland (North-eastern region)	N_t = 9 3 samples from 3 different farms$^{(F1\ to\ F3)}$	Poland (North-eastern region)	ZEN	Intestine Liver Ovary Muscle	Intestine = n.d.^{F1}; <2.0^{F2}; <2.0^{F3} Liver = n.d. ^{F1}; <2.0^{F2}; nd^{F3} Ovary = <2.0^{F1}; =7.1 ± 3.2^{F2}; <2.0^{F3} Muscle = n.d.$^{F1\ to\ F3}$ Water = n.d.$^{F1\ to\ F3}$	HPLC	
Woźny *et al.* (2017)	Poland 2013^{T1}, 2014^{T2}, 2015^{T3}	n = 35 (acquired from hatcheries)^{AQH} n = 6 (from supermarket)^{S}	Norway Poland	ZEN, α-ZEL, β-ZEL	Ovary^{Ov} Oocytes^{Oo} Salted roe^{Sr}	ZEN, α-ZEL, β-ZEL^{Ov} = Detected in 4/4 samples$^{T2;\ Om;\ Sf}$ and in 1/6 samples$^{T3;\ Om;\ Ss}$ ZEN, α-ZEL, β-ZEL^{Oo} = Detected in 5/13 samples$^{T2;\ Au,\ Cl,\ Cl,\ Hm,\ Om,\ Sf,\ Sg}$; in 5/6 samples$^{T2;\ Cl,\ Ok,\ Om,\ Sf}$ and in 2/6 samples$^{T3;\ Ok,\ Om,\ Ss}$ ZEN, α-ZEL, β-ZEL^{Sr} = Detected in 0/1^{T1}; in 2/3 samples$^{T3;\ Ok,\ Om}$ and in 2/2 samples$^{T3;\ Ok,\ Om}$ *^{1}α-ZEL^{Ov} = 14.5$^{T2;\ Om}$ *^{1}α-ZEL^{Ov} = 12.6$^{T2;\ Sf}$ All mycotoxin levels detected below LOD (ZEN, α-ZEL, and β-ZEL were 5.0, 3.0, and 12.0 μg kg^{-1}) except *^{1}	HPLC-FLD	Species sampled: *Acipenser oxyrinchus*^{Au} *Coregon lavaretus*^{Cl} *Ctenopharyngodon idella*^{Ci} *Hypophthalmichthys molitrix*^{Hm} *Oncorhynchus mykiss*^{Om} *Salvelinus fontinalis*^{Sf} *Silurus glanis*^{Sg} *Oncorhynchus keta*^{Ok} *Salmo salar*^{Ss}

Reference entries are alphabetically ordered by publication first author. Superscript letters give extra information; they are only valid for the same row.

General abbreviations: HPLC, High-performance liquid chromatography; HPCL-FLD, high-performance liquid chromatography: fluorescence detection; LC–MS/MS, Liquid chromatography-tandem mass spectrometry; n.d., not detected.

Mycotoxins: BEA, beauvericin; ENA, enniatins; ENA1, enniatin A1; ENA2, enniatin A2; ENB, enniatin B; ENB1, enniatin B1; ZEN, zeralenone; α-ZEL, alpha-Zearalenol; β-ZEL, beta-Zearalenol.

There is the need to perform studies for the main EU farmed fish species in order to establish acceptable feed mycotoxin levels for farmed fish (for both fish and consumer safety), but also to actively survey possible mycotoxin deposition in imported aquaculture foods.

Woźny *et al.* (2013) analysed ZEN in rainbow trout from farms based in the north-eastern region of Poland. ZEN was present at non-quantifiable levels (<2.0 μg kg^{-1}) in most of the tissues analysed (intestine, liver and ovary) and detectable at quantifiable levels in the muscle and surrounding water. From 2013 to 2015, Woźny *et al.* (2017) surveyed ovary, oocytes and salted roe samples from different fish species collected directly at hatcheries or bought in supermarkets. The authors analysed the samples for the presence of ZEN, α-ZEL and β-ZEL. Generally, in most of the samples analysed mycotoxins were below the detection limits (LOD for ZEN, α-ZEL and β-ZEL were 5.0, 3.0 and 12.0 μg kg^{-1} respectively). The exceptions were α-ZEL in ovary samples (14.5 μg kg^{-1}) of *O. mykiss* and α-ZENL also in ovary samples (12.6 μg kg^{-1}) of *Salvelinus fontinalis* both sampled in 2014. The studies reported by Woźny *et al.* (2013, 2017) are also extremely important and highlight the need for guidance values for the amount of ZEN in aquafeeds for fish health and reproductive performance, but also to avoid carry-over risk to human consumers.

Although it did not investigate fish originating from aquaculture, it is important to highlight the recent study published by Slawomir Gonkowski *et al.* (in press). Slawomir Gonkowski *et al.* (2018) evaluated the deposition of ZEN in sun-dried kapenta fish, which is one of Zambia's major staple foods. This small planktivorous fish is caught in Lake Kariba, sun-dried and sold in local markets. Although the source of the ZEN deposition is not known, the study revealed that levels of ZEN in sun-dried kapenta fish fluctuated from about 27 μg kg^{-1} to above 53 μg kg^{-1}. Occurrence of ZEN in sun-dried kapenta fish, highlights that carry-over guidelines cannot be assumed only for farmed animals as species and local consumption habits pose mycotoxin-related risks to wider seafood products.

Further considerations

Despite the effort to document mycotoxin occurrence in aquaculture feeds, we are still far from having a good overview on this topic. One of the big challenges is the large number of aquaculture-farmed species, and the impossibility to extrapolate occurrence results from one species to another. Moreover, different species, even in same trophic level, tend to be fed with different raw materials based on local availability and price. This leads to a huge difficultly in having a good overview of mycotoxin occurrence for all aquaculture species or even for a certain region. Nevertheless, knowledge about mycotoxin occurrence in aquaculture commodities could increase significantly if we could better use the available occurrence data from livestock. Surveys on mycotoxin occurrence in plant meals worldwide are frequently available, and this information can be used, at least, to theoretically model the risk of plant feedstuffs included in aquafeeds. However, a fundamental problem is the lack of detailed labelling information regarding ingredient inclusion by (percentage) weight. Therefore, an improvement in labelling policy would help to identify and map sources of mycotoxin inclusion in animal feed, avoiding extra costs for testing mycotoxin levels in finished feeds. Therefore, a close collaboration with the agricultural and livestock sectors to understand the occurrence of mycotoxins in plant meals, might also help to improve our knowledge on mycotoxin conveyance to aquafeeds.

Mycotoxins conveyed from land animals and aquaculture by-products cannot be despised, especially in countries were mycotoxin occurrence might be poorly legislated. The identification of mycotoxins in shrimp head meal or chicken droppings highlights the possible bio-amplification through the food chain.

To our knowledge not yet addressed in an aquaculture context, is the potential for mycotoxins to contaminate water, especially taking into account water stable mycotoxins and closed or semi-closed aquaculture systems. Bucheli *et al.* (2008) evaluated the presence of ZEN and DON in Swiss rivers, confirming the presence of both mycotoxins at levels ranging from 23 ng L^{-1} to 4.9 μg L^{-1} for DON and 35 ng L^{-1} for ZEN. Bucheli *et al.* (2008) highlighted the possibility of mycotoxins as water contaminates, which in the aquaculture context might be extremely relevant. The mycotoxin leach from aquafeed to system water, especially of highly water-soluble mycotoxins in slow feeding species, for example DON and FUM in shrimp feed, and the water stability of excreted mycotoxins and metabolites, which might have potential to accumulate, especially in low water hydrodynamics and low renovation rate aquaculture systems, should be urgently addressed.

Conclusion

The available carry-over studies indicate that deposition of mycotoxins into edible tissues may be higher than in terrestrial species and it is therefore imprudent to assume the same transfer factors for aquaculture species as for livestock species. In general, aflatoxins seem to be particularly prone to deposition in several fish and shrimp tissues representing a risk for human consumption, especially in species that are eaten as a whole. Ochratoxin A occurrence in aquafeeds has been described as very low. While its deposition in tissues has been reported for some aquaculture species, its rapid elimination decreases the risk for human consumption as the fasting period before slaughter can be safely used as a

depuration period. Nonetheless, it is important to make the industry aware of its possible deposition. Deoxynivalenol and fumonisins are some of the most frequently occurring mycotoxins in feeds, and they are occasionally detected at high levels. So far, for the species described, DON and FUM deposition in tissues seems low. However, DON elimination from the muscle takes a relatively long time, much longer than the depuration/fasting period. The presence of enniatins in aquaculture food products highlights the possibility that other *Fusarium* metabolites might be more prone to bioaccumulation than the most common frequently analysed *Fusarium* mycotoxins. The presence of enniatins in aquaculture foods highlights the need to understand its potential impact to human food safety.

Regarding ZEN, the potential for deposition in the ovaries and to a lesser extent in oocytes was shown. For the studied species, ZEN can reach considerable levels in the ovaries. No studies are available yet for tropical species. It would be important to investigate whether carry-over of ZEN to ovaries occurs in tropical species as well, as for many of these species, gonads are considered gourmet snacks, representing a direct risk to human health.

While there are many important aquaculture species not investigated yet, it is clear that some mycotoxins are prone to deposition in the tissues of certain aquaculture species. It needs to be considered that in aquaculture species, mycotoxin biotransformation and tendency for deposition in tissues varies greatly depending on factors such as development stage, sex, exposure period and rearing environment.

Due to the use of increasing levels of plant meals in aquafeeds and together the possible mycotoxin increase due to climate change, it is essential to develop more studies on the impact of mycotoxins and metabolites on farmed species with consequent risk assessment of food safety from mycotoxin-contaminated aquafeeds.

Regulation limits for mycotoxins in feeds might need to take into account particular aquaculture species or the sector as a whole. Mycotoxin limits need to take into consideration animal health and welfare but also human health. Particular attention needs to be focused on aquaculture edible tissues and regional guidance limits should be advised depending on local mycotoxin occurrence and the edible tissues consumed. Risk assessment of imported aquaculture foods needs to take into account the mycotoxin occurrence, especially in those products imported from highly mycotoxin contaminated regions, or regions known to use potentially contaminated animal by-products.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Overall transfer of mycotoxins from feed to eggs, whole milk, meat and edible offal.

• CHAPTER 2 - Effects of deoxynivalenol exposure time and contamination levels on rainbow trout was based on:

Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

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APPLIED STUDIES

Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

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The trend toward using plant-based ingredients in aquafeeds is set to intensify; however, mycotoxin contamination might be a challenge. Two diets, with deoxynivalenol (DON) levels of 1,166 μg/kg (1.1 DON) and 2,745 μg/kg (2.7 DON), were prepared for short-term DON exposure (50 days). A third diet with a low DON level of 367 μg/kg (0.3 DON) was prepared for long-term DON exposure (168 days). Ingestion of DON by trout during both short-term/high-dosage exposure (50 days; 1,166 μg/kg and 2,700 μg/kg DON) and long-term/low-dosage exposure (168 days; 367 μg/kg DON) impacted growth performance and, to a lesser extent, liver enzyme parameters (2.7 DON). Histopathology showed mild to moderate changes in the liver but not in the other sampled tissues (intestine and kidney). Despite these effects, short-term exposure of rainbow trout to high doses of DON did not result in increased susceptibility to *Yersinia ruckeri*. In both the short- and long-term studies, the effects of DON showed a high interindividual variability. The present study confirms that subclinical levels of mycotoxins affect rainbow trout. The effects of such low mycotoxin levels could be masked by other production challenges while still negatively affecting productivity.

KEYWORDS

hepatocyte hyalinization, mycotoxins, *Oncorhynchus mykiss*, pathogen susceptibility

1 | INTRODUCTION

In aquaculture, the trend to replace expensive animal-derived proteins, such as fishmeal, with more economical and sustainable plant protein sources has increased the probability of mycotoxin contamination in aquaculture feeds. According to Tacon, Hasan, and Metian (2011), plant-based ingredients already represent the major dietary protein source used in feeds for lower-trophic-level fish species, such as tilapia, carp, and catfish. These ingredients also

account for the second major source of dietary protein and lipids after fishmeal and fish oil in the feed of shrimp and high-trophic-level fish species. Various plant sources have been used for salmonids but at lower inclusion levels than feed destined for lower-trophic species. In most aquaculture species, plant protein choice and selection are based on a combination of local market availability, cost, and the nutritional profile (including antinutrient content and level) of the plant meal in question (Davis & Sookying, 2009; Gatlin et al., 2007; Krogdahl, Penn, Thorsen, Refstie, & Bakke, 2010).

The mycotoxin contamination of finished feeds and raw materials used in aquaculture as well as the negative effects of mycotoxins on aquatic species, particularly rainbow trout, *Oncorhynchus mykiss*, have been highlighted in recent publications (Gonçalves, Navarro-Guillén, et al., 2018; Hooft & Bureau, 2017; Hooft, Elmor, Encarnação, & Bureau, 2011; Ryerse, Hooft, Bureau, Hayes, & Lumsden, 2015; Tola et al., 2015). However, mycotoxin contamination is not generally assessed in commercial aquafeeds or plant meals used to manufacture these feeds. Consequently, we do not have accurate estimates of the mycotoxin contamination levels in these commodities.

Few studies are currently available concerning mycotoxin occurrence in aquaculture plant meals and finished feeds. Gonçalves, Naehrer, and Santos (2016) reported that deoxynivalenol (DON) was present in 68% of analyzed samples (shrimp and fish, sampled in Asia and Europe in 2014) at average contamination levels of 162 μg/kg and maximum levels of 413 μg/kg. More recently, Gonçalves, Hofstetter, Schatzmayr, and Jenkins (2018) observed that contamination patterns for shrimp and fish feeds were slightly different, which likely reflects the type of commodity used for the different species. The authors observed that shrimp feeds were generally contaminated with low levels of DON, with the exception of some diets (contamination ranging from 329 μg/kg to 2,287 μg/kg of DON). In the case of fish feeds, samples were contaminated mainly by DON, up to a maximum level of 396 μg/kg, and were co-contaminated with other mycotoxins.

Trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with the initiation, elongation, and termination stages of this process (Kumar, Roy, Barman, & Anand, 2013). Knowledge of the effects of DON on aquatic species has increased recently (Gonçalves, Hofstetter, et al., 2018; Hooft & Bureau, 2017; Hooft et al., 2011; Matejova et al., 2015; Ryerse et al., 2015; Tola et al., 2015), and studies on rainbow trout suggest that DON has a detrimental effect on feed intake (FI), weight gain, and feed efficiency (Hooft et al., 2011; Ryerse et al., 2015). Curiously, no effect has been detected on the immune status of animals fed with DON (Matejova et al., 2015; Matejova, Svobodova, Vakula, Mares, & Modra, 2017; Ryerse et al., 2015).

In general, the effects of mycotoxicoses vary greatly depending on a variety of factors, including nutritional and health status prior to exposure, dose and duration of exposure, age, species, and infection route. In addition, the lack of reliable clinical signs or parameters (including biomarkers) to correctly diagnose the ingestion of DON by aquatic species makes mycotoxin risk management in aquaculture very challenging.

The aim of the present study was to evaluate the effect of DON on rainbow trout under two different scenarios: first, the effect of short-term feeding of high levels of DON (50 days; 1,166 μg/kg DON and 2,745 μg/kg DON), and second, the effects of long-term feeding of low levels of DON (168 days; 367 μg/kg DON). Moreover, we aimed to investigate the manifestation of clinical signs due to the ingestion of DON by inspecting several organs and tissues normally affected by the consumption of mycotoxins.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

The experimental diets were formulated to be isoenergetic (22.20 kJ/g dry matter [DM]), isoproteic (52.20% DM), and isolipidic (17.90% DM; Table 1). All diets were formulated with the same ingredients. Marine-derived ingredients (fishmeal and fish oil) represented 22.45% DM of the diet, whereas plant raw materials represented 59.70% DM of the diet. All ingredients were finely ground (hammer mill, 0.8-mm sieve), mixed, and then extruded (twin screw extruder, 2.0-mm pellet size; SPAROS, Portugal).

TABLE 1 Experimental control diet ingredients and proximate composition

Ingredients	Control (%)
Fishmeal 60[1]	14.00
Fishmeal Super Prime[2]	12.45
Soy protein concentrate[3]	15.00
Wheat gluten[4]	12.30
Corn gluten meal[5]	8.00
Soybean meal[6]	6.00
Wheat meal[7]	6.40
Corn meal[8]	10.00
Fish oil[9]	10.00
Soy lecithin[10]	2.00
Antioxidant[11]	0.30
Monocalcium phosphate[12]	1.50
L-Lysine[13]	0.50
DL-Methionine[14]	0.50
Vitamin E[15]	0.05
Vitamin and mineral premix[16]	1.00
Proximate composition (% dry matter [DM])	
Dry matter	91.7 ± 0.0
Crude protein	52.2 ± 0.1
Crude fat	17.9 ± 0.0
Ash	9.3 ± 0.0
Gross energy, kJ/g DM	22.2 ± 0.0

[1]COFACO 60: 62.3% crude protein (CP), 8.4% crude fat (CF); COFACO, Portugal, Aveiro. [2]Super Prime: 67.4% CP, 8.2% CF; EXALMAR, Peru, Lima. [3]Soycomil P: 63% CP, 0.8% CF; ADM, The Netherlands, Koog aan de Zaan. [4]VITAL: 83.7% CP, 1.6% CF; ROQUETTE Frères, France. [5]Corn gluten meal: 61% CP, 6% CF; COPAM, Aveiro, Portugal. [6]Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF; CARGILL, Madrid, Spain. [7]Wheat meal: 10.2% CP, 1.2% CF; Casa Lanchinha, Moita, Portugal. [8]Corn meal: 8.1% CP, 3.7% CF; Casa Lanchinha, Moita, Portugal. [9]SAVINOR, covelas, Portugal. [10]Lecico P700IPM; LECICO GmbH, Hamburg, Germany. [11]Paramega PX; Kemin Europe NV, Brussels, Belgium. [12]Monocalcium Phosphate (MCP): 22% P, 18% Ca; Fosfitalia, Faenza, Italy. [13]Lysine HCl 99%; Ajinomoto Eurolysine SAS, Paris, France. [14]DL-Methionine 99%; EVONIK DEGUSSA GmbH, Essen, Germany. [15]ROVIMIX E50; DSM Nutritional Products, Aesh, Switzerland. [16]PREMIX Lda, Neiva, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthothenate, 100 mg; choline chloride, 1,000 mg; betaine, 500 mg. Minerals (g or mg/kg diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

The ingredients used to formulate the diets were subjected to liquid chromatography–tandem mass spectrometry (MS), high-performance liquid chromatography (HPLC)-MS/MS-based multimycotoxin analysis (University of Natural Resources and Life Sciences, Center for Analytical Chemistry Department IFA, Austria), as described by Streit et al. (2013). The method covered major type A and B trichothecenes, zearalenone, fumonisins, aflatoxins, and ochratoxins. For the purpose of data analysis, nondetect levels were based on the limits of detection of the method used for analysis. The detected concentrations of major mycotoxins and of a selection of other fungal metabolites are listed in Table 2.

Diets with three different levels of DON were prepared by adding DON (Romer Labs Diagnostic GmbH, Austria) to the feed during diet ingredient mixing. Two diets, with DON levels of 1,166 μg/kg (1.1 DON) and 2,745 μg/kg (2.7 DON), were prepared for short-term DON exposure (50 days). A third diet with a low DON level of 367 μg/kg

TABLE 2 Multimycotoxin analysis of experimental diets

Analyte	Concentration (μg/kg)	Analyte	Concentration (μg/kg)
Major mycotoxins		Other *Fusarium* metabolites	
Aflatoxin B1	<LOD	15-Hydroxyculmorin	48.33
Zearalenone	11.44	Culmorin	69.87
Deoxynivalenol (DON)	<LOD	Equisetin	10.39
Fumonisin B1	<LOD	Fusaric acid	65.56
Fumonisin B2	25.05	*Penicillium* metabolites	
Fumonisin B4	16.11	Brevianamid F	194.30
Ochratoxin A	<LOD	Mycophenolic acid	88.91
Sum of ergot alkaloids	0.72	Rugulusovin	244.20
		Other *Aspergillus* metabolites	
Zearalenone metabolites		Tryptophol	28.90
Zearalenone-sulfate	32.62	Other metabolites	
		Cyclo(L-pro-L-Val)	1,631.00
		Cyclo(L-pro-L-Tyr)	2,004.00
DON	Target concentration	Analyzed concentration	
Control	0.0	0.0	
1.1 DON	1,500	1,166 ± 140	
2.7 DON	3,000	2,745 ± 330	
0.3 DON	400	367 ± 66.80	

Note. Limits of detection (LOD) for aflatoxin B1 = 0.3 μg/kg. For DON and ochratoxin A, detection limit are 10, 50, and 0.2 μg/kg and for fumonisin B1 the detection limit are 25 μg/kg. Five samples per diet were analyzed.

(0.3 DON) was prepared for long-term DON exposure (168 days). All diets were dried at 45°C for 12 hr after the addition of DON and were stored at 4°C until use.

Contamination levels were chosen taking into account previous literature on the effect of DON on rainbow trout (Hooft et al., 2011; Matejova et al., 2014; Matejova et al., 2015; Ryerse et al., 2015) as well as the reported DON levels in worldwide finished feed samples (Barbosa et al., 2013; Gonçalves, Hofstetter, et al., 2018; Gonçalves, Navarro-Guillén, et al., 2018; Gonçalves et al., 2016; Gonçalves, Schatzmayr, Hoffstetter, & Santos, 2017; Greco, Pardo, & Pose, 2015). The long-term exposure to DON attempts to mimic the most recently reported levels of DON in finished feeds (Gonçalves, Hofstetter, et al., 2018; average of 82.87 μg/kg and maximum of 396 μg/kg). However, we are aware that reports of mycotoxin occurrence in European aquaculture finished feeds are still very limited, and levels reported may vary annually (e.g., average DON contamination of 160.86 μg/kg in 2014, 165.61 μg/kg in 2015, and 87.87 μg/kg in 2016; Gonçalves et al., 2016; Gonçalves et al., 2017; Gonçalves, Hofstetter, et al., 2018). Generally, Asian aquafeed samples present higher DON levels compared with European aquafeed samples.

2.2 | Fish and experimental conditions

This study was approved by the institutional ethics committee and the national authority according to §26 of Law for Animal Experiments, Tierversuchsgesetz 2016—TVG 2012 under No. BMWFW-68.205/0135-WF/V/3b/2014. Rainbow trout, *O. mykiss*, originating from a farm with no prior history of yersiniosis was used in both experiments. On arrival, the kidneys of 10 fish were sampled, and their infection-free status was confirmed by culture-based analysis and polymerase chain reaction (PCR)-based analysis using *Yersinia ruckeri*-specific primers (del Cerro, Marquez, & Guijarro, 2002).

2.3 | Short-term exposure to DON

For the experiment with short-term exposure to DON, 180 fish (14.10 ± 0.05 g) were randomly allocated to three feeding groups in quadruplicate and given either standard feed (control [CTRL]), feed contaminated with 1,166 μg/kg DON (1.1 DON), or feed contaminated with 2,745 μg/kg DON (2.7 DON). Each aquarium of 85 L was supplied by a flow-through system with a temperature of 15.47 ± 0.14°C, oxygen concentration of 8.73 ± 0.12 mg/L, and pH of 7.53 ± 0.04, with 0.0 ± 0.0 mg/L total ammonia nitrogen, nitrites, and nitrates. The fish were hand fed the prepared diets (CTRL, 1.1 DON or 2.7 DON) thrice per day near satiety for 50 days prior to performing the *Y. ruckeri* challenge.

2.4 | Long-term exposure to DON

For the long-term exposure experiment, 120 fish weighing 89 ± 8 g were randomly allocated and distributed among eight tanks, each with a volume of 1 m^3, supplied by a flow-through system with a water temperature of 18.6 ± 1.0°C, oxygen concentration of 8.56 ± 0.26 mg/L, and pH of 7.35 ± 0.35. Each tank contained 15 fish that were fed restrictively (2.5% of the average body mass) with either control feed (CTRL, four tanks) or the control feed supplemented with 367 μg/kg DON (0.3 DON, four tanks) for 168 days. The same quantity of feed (2.5% of the average body mass) was distributed in each tank by hand feeding and was adjusted after intermediate weighing periods (at 37, 62, 92, and 125 days). Five fish per replicate tank were subjected to moderate anesthesia (tricaine methanesulfonate [MS-222]; Sigma-Aldrich Co., LLC, Bellefonte, PA) at a dose of 0.7 g/L, and a blood sample was collected by puncture of the caudal vein with a heparinized syringe at the beginning of the trial and at 62 and 125 days. Part of the blood sample was used for the determination of hematocrit, which was determined for five fish per treatment. Blood was transferred into hematocrit capillary tubes (Hirschmann), the tubes were then centrifuged at 160 g for 5 min (Hettich Haematokrit 200 Hettich, Buckinghamshire, England), and the percentage of red blood cells to sera was measured. The remaining part of the blood sample was centrifuged at 1,590 *g* for 10 min, after which the plasma (i.e., the supernatant fraction) was transferred to Eppendorf tubes, snap frozen in liquid nitrogen, and stored at −80°C until subsequent analysis of total protein. Total protein was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. All measurements were performed in a Synergy HT multimode microplate reader (BIOTEK, Winooski, VT).

2.5 | Growth performance

All fish, in both the short- and long-term exposure experiments, were weighed to determine the initial individual body weight at the start of the experiments. In the short-term exposure study, the fish were weighed individually at the end of the 50-day period, and their total length was measured and recorded. FI was recorded daily. In the long-term exposure study, the fish were weighed individually after 37, 62, 92, 125, and 168 days.

The following calculations were made in both the experiments.

The thermal-unit growth coefficient (TGC) was expressed as the growth rate and was calculated for each aquarium as (100 × [FBW 1/3 − IBW 1/3]/Σ [Temp (°C) × number of days]), where FBW is the final body weight (g/fish) and IBW the initial body weight (g/fish).

The feed conversion ratio (FCR) was calculated as crude FI/weight gain, where FI = total dry feed/number of fish.

The protein efficiency ratio (PER) was calculated as weight gain (g)/protein intake (g).

The specific growth rate (SGR) was calculated as ([ln final weight − ln initial weight]/time in days) × 100.

Fulton's condition factor (CF), K, was also used to measure individual fish health: $K = 100(BW/L^3)$, where BW is the whole body wet weight (g) and L is the length (cm). A factor of 100 was used to transform K to approximate a value of one.

2.6 | Liver enzymes

In the short-term/high DON exposure experiment, five fish from each aquarium were sampled at the end of the experiment (50 days) for analysis of liver enzymes in blood. In the long-term/low DON exposure study, five fish from each aquarium were sampled on Day 62 and on Day 125. The fish were anesthetized by immersion in MS-222 (Sigma-Aldrich Co., LLC, Bellefonte, PA) at a dose of 0.7 g/L prior to blood collection. Blood samples were analyzed to measure the activities of lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate aminotransferase (AST) using a Spotchem EZ SP-4430 reader and Spotchem II GPT/ALT, Spotchem II LDH, and Spotchem II GOT/AST kits (all products from Arkay, Amstelveen, Netherlands).

2.7 | Histological examination

For the short-term/high DON exposure study, organs were sampled from 10 fish prior to the *Y. ruckeri* challenge and at the time of termination. The intestine, spleen, liver, and kidneys (head and trunk kidney) of the fish were removed and fixed in 10% buffered formalin for 48–72 hr. The samples were embedded overnight in paraffin using a Histo-Master (Formafix, Düsseldorf, Germany). Sections (3–4 μm thick) were cut from each paraffin block and were left to dry overnight at 37°C before being stained with hematoxylin and eosin.

The slides were evaluated under a light microscope (Nikon Eclipse E400, Feasterville, PA). The following were examined: intestine (number of mucous cells in mucosa), liver (hepatocyte vacuolation, hepatocyte hyalinization, single-cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation), and kidney (number of pigmented macrophage centers). To evaluate the number of cells, three high-power fields were counted per slide.

2.8 | Bacterial preparation

As a pretrial to the challenge test, five groups of 10 fish each were challenged by immersion with *Y. ruckeri* isolate 7959/11 to determine the appropriate infectious dose. *Y. ruckeri* isolate A7959/11 is a clinical isolate that originated from an outbreak at an Austrian trout farm in 2011. This isolate was kept at −80°C on beads until 3 days prior to the start of the experiment. It was then inoculated on a blood agar plate and incubated at 22°C. After 48 hr, a single colony was inoculated into 7.5 mL of brain heart infusion (BHI) broth and was incubated in a shaking incubator at 20°C with rotation at 150 rpm. After 10 hr, the cultures were evaluated by eye, and 2.5 mL was sampled from one culture and used to inoculate a 1.5-L BHI broth. This broth was then incubated for approximately 12 hr at 20°C with shaking at 150 rpm.

2.9 | Infection trial in the short-term exposure study

After 50 days, each feeding group of the short-term/high DON exposure study was further divided into two groups: two of the aquaria were infected with *Y. ruckeri*, while fish in the two other aquaria were mock-infected with uninoculated broth. In total, 90 fish were infected and 90 were mock-infected. The infection procedure was adapted from that described for *Aeromonas salmonicida* (Menanteau-Ledouble et al., 2017). Briefly, bacteria were grown overnight in 1.5 L of BHI broth and their concentration was determined by measuring the optical density at a wavelength of 600 nm (optical density 600) per mL. Water circulation in the aquarium was interrupted, and the water volume was lowered to 50 L. The bacterial culture (2 mL) was added to each of the aquaria, yielding a final concentration of 2×10^4 CFU/mL. The fish remained in the solution for 2 hr, after which the water was progressively returned to its normal level and the circulation was reopened. The fish were monitored at least twice daily. Mortalities were recorded, and dead and moribund fish were immediately removed from the tanks. Moribund fish were euthanized by prolonged immersion in a solution of 1 g/L of MS-222, and the kidney of the fish was sampled for microbial re-isolation of the pathogen on an agar plate. The colonies growing on these plates were examined and confirmed to be

Y. ruckeri based on their morphologies. Furthermore, one in five isolates was selected; its genomic DNA was isolated using a Qiagen DNeasy kit, and PCR was performed using *Y. ruckeri* specific primers (del Cerro et al., 2002). The surviving fish overcame the infection 17 days postinfection, at which point the challenge was terminated. All remaining fish were euthanized by prolonged immersion in a solution of MS-222 (1 g/L of water), weighed, measured, and examined for gross clinical signs of enteric red mouth syndrome (oral congestion, hemorrhages or petechia, exophthalmia and ocular hemorrhages, ascites in the abdominal cavity, enlarged spleens and hemorrhages or petechia in the internal organs, and bloody intestines or adipose tissues).

2.10 | Clinical signs

During both the experiments, gross clinical signs were assessed by visual examination of the fish at the time of termination. Lesions (hemorrhages and ulcerations) on the skin were recorded, as were any obvious abnormalities such as a protruding anal papilla. The state of the gills was recorded as well as the presence of anemia, hemorrhages, or necrosis.

The fish were examined internally for any abnormalities. In particular, record was made of congestions, petechia, or hemorrhages of the internal organs. The color of the liver and the size of the spleen were assessed as was the general health of the intestine (in particular, the presence of congestion, hemorrhage, or intussusception was determined).

2.11 | Statistical analysis

All parameters such as the final weight, SGR, PER, FI, FCR, CF, TGC, LDH, ALT, and AST were subjected to ANOVA in SPSS 21 for Windows (IBM Corp., Armonk, NY). One-way ANOVA was performed, and differences between the means were tested by Tukey's multiple range test. The Shapiro–Wilk's test was used to analyze the normality, and homogeneity of variances was tested using Levene's test. Data analyzed did not violate the assumption of equal variances and showed a normal distribution. All parameters expressed as percentages were subjected to arcsin square root transformation. Additionally, one-way ANOVA was performed to analyze the histological differences in the intestine (number of mucous cells in mucosa) and liver (single-cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation) between the DON dietary treatments and controls.

Following the challenge, survival curves were constructed for each treatment, and Kaplan–Meier and odds ratio analyses were performed using SPSS v.20 (IBM Corp., Armonk, NY) and MedCalc (Microsoft, Redmond, WA).

The level of significance was set at $p < .05$, and the results are presented as the mean ± *SD* (*SD* of the mean).

3 | RESULTS

3.1 | Experimental diets

The four experimental diets were formulated to be isoenergetic (22.20 kJ/g DM), isoproteic (52.20% DM), and isolipidic (17.90% DM) and to meet all the nutrient requirements for the species examined in the study. There was no significant difference ($p > .05$) between treatments regarding the nutritional composition of the experimental diets. Analysis of the feed to confirm mycotoxin levels showed DON contamination was successfully achieved, although observed levels were slightly lower than intended (Table 2). Other metabolites/toxins were found in the basal diet (common to all experimental groups) due to natural contamination of the plant raw materials used to formulate the diet (Table 2). Generally, these metabolites/toxins, produced mainly by *Fusarium* and *Aspergillus*, were at levels below 100 μg/kg. Regarding the *Penicillium* toxins, brevianamide F and rugulusovin were found at levels of 194 and 244 μg/kg, respectively. Fungal and bacterial metabolites were also detected in the experimental diets, namely cyclo (L-Pro-L-Val) and cyclo (L-Pro-L-Tyr) at relatively high concentrations (1,631 and 2,004 μg/kg, respectively).

TABLE 3 Growth performance parameters determined in the short-term/high deoxynivalenol (DON) dosage study

	Final weight (g)	SGR (%/day)	PER	FI (g/fish)	FCR	CF	TGC
Control	101.36 ± 19.81 [a]	2.52 ± 0.07 [a]	2.17 ± 0.05	81.21 ± 4.71 [a]	0.98 ± 0.07	1.42 ± 0.12 [ab]	0.113 ± 0.005 [a]
1.1 DON	95.37 ± 19.20 [a]	2.46 ± 0.06 [a]	2.01 ± 0.13	81.65 ± 3.78 [a]	1.03 ± 0.07	1.46 ± 0.13[b]	0.109 ± 0.004[a]
2.7 DON	79.91 ± 16.54 [b]	2.20 ± 0.09 [b]	2.01 ± 0.07	64.03 ± 2.87 [b]	1.05 ± 0.04	1.39 ± 0.12 [a]	0.094 ± 0.005 [b]
1-way ANOVA							
p value	<.001	<.001	.096	<.001	.423	.033	.001

Note. Data are presented as mean ± *SD*. Values in the same column with different letters are significantly different ($p < .05$). CF = condition factor; FCR = feed conversion ratio; FI = feed intake; PER = protein efficiency rate; SGR = specific growth rate; TGC = thermal-unit growth coefficient.

3.2 | Growth performance

3.2.1 | Short-term DON exposure

The results showed that rainbow trout was sensitive to the DON levels tested (Table 3).

The presence of 2,700 μg/kg DON in the diet led to a significant decrease ($p < .001$) in FI. The same treatment (2.7 DON) also resulted in a significant decrease in the final weight (79.91 ± 16.54 g; $p < .001$), SGR (2.20 ± 0.09%/day; $p < .001$), TGC (0.094 ± 0.005; $p < .001$), and CF (1.39 ± 0.12; $p < .033$) compared to the controls (final weight = 101.36 ± 19.8 g; SGR = 2.52 ± 0.07%/day; TGC = 0.113 ± 0.005; and CF = 1.42 ± 0.12). Observations of the feeding behavior of the DON-fed groups confirmed that the fish initially accepted the feed, and a reduction in FI was progressively established. We therefore assumed that the lower FI in the DON-fed groups compared to the control group was probably not due to the unfavorable organoleptic properties of DON-contaminated feed.

3.2.2 | Long-term DON exposure

In the long-term exposure study, the fish that received the contaminated diet also showed lower farming performances (FBW, FCR, and SGR) compared to the control. These differences increased over time (Figures 1, 2 and 3) and after 168 days of exposure to 367 μg/kg DON, fish that ingested DON presented a final weight of 487.40 g compared to 593.63 g in the control group ($p = .053$; Figure 1). However, these differences were never statistically significant.

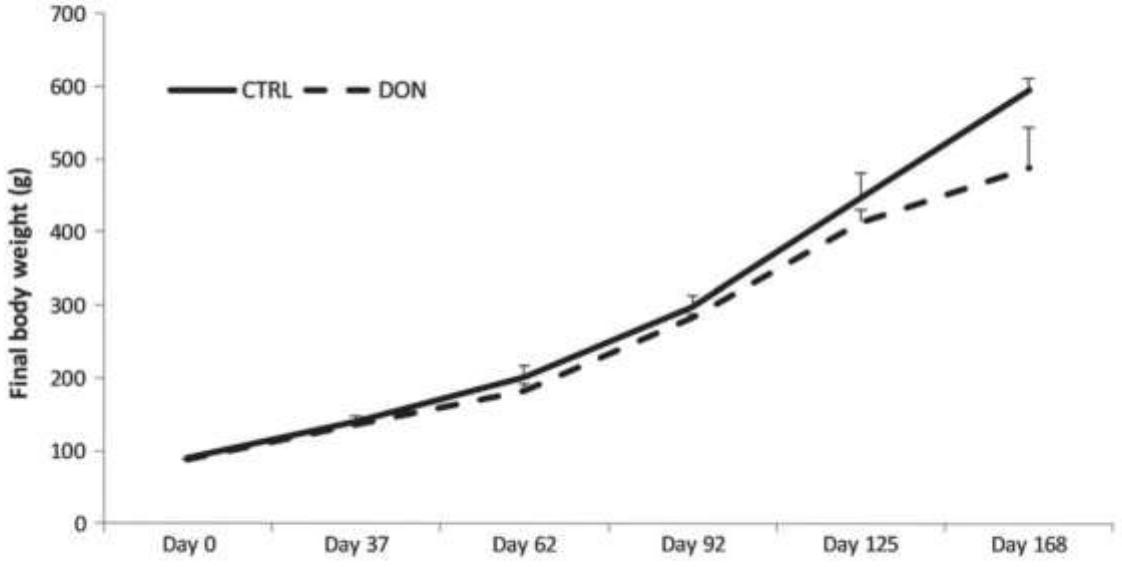

FIGURE 1 Growth curve representing the average weight of the fish during the long-term experiment

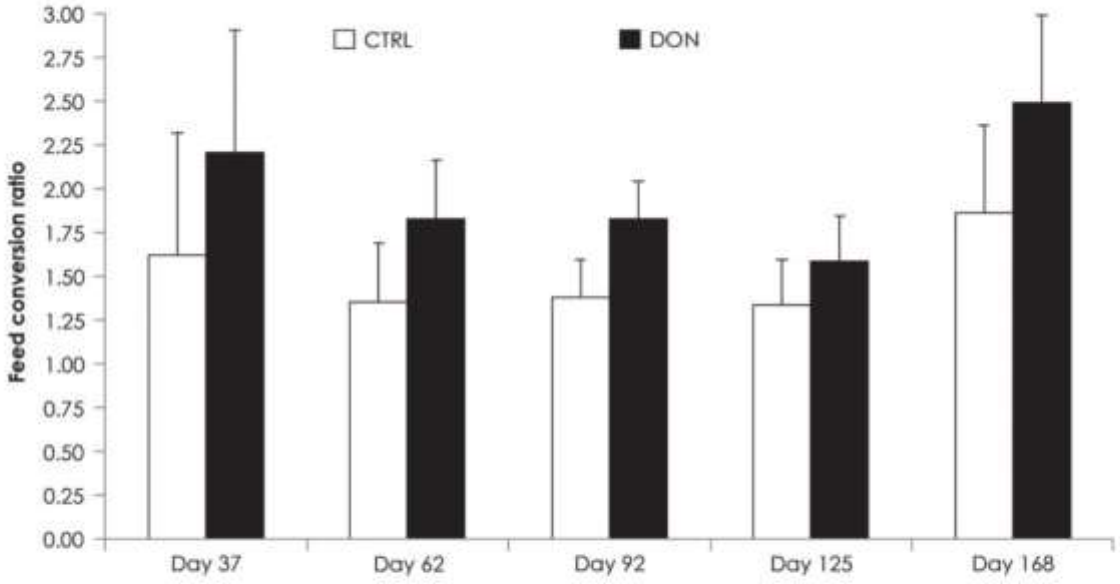

FIGURE 2 Feed conversion ratio at different sampling time points. Values are displayed as average ± *SD*

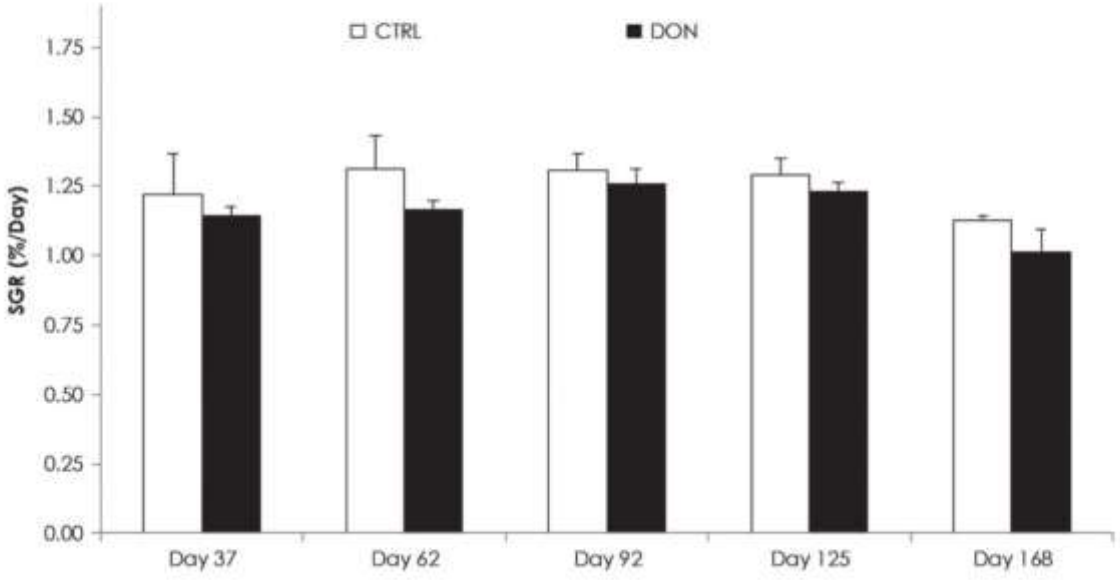

FIGURE 3 Specific growth rate at different sampling time points. Values are displayed as average ± *SD*

A similar pattern of lower performance in the DON-fed animals was observed for FCR (Figure 2) and SGR (Figure 3): Animals fed the control diet presented an FCR of 1.86 compared to 2.50 for DON-fed animals. PER was generally lower for animals that were fed DON and was significantly lower on day 92 (p = .044) and day 168 (p = .050; Table 4). FI was generally higher for animals that were fed DON and was significantly higher on day 62 (p = .041; Table 5).

3.3 | Histology

In the short-term exposure study, among the 2.7 DON groups, 2 of 10 animals showed mild to moderately hyalinized hepatocytes. In one trout, multiple areas of necrosis with scattered hemorrhages were present

TABLE 4 Protein efficiency rate at different sampling time points for the long-term/low deoxynivalenol (DON) dosage experiment

	Day 37	Day 62	Day 92	Day 125	Day 168
Control	1.15 ± 0.17	1.38 ± 0.18	1.34 ± 0.11	1.38 ± 0.13	0.99 ± 0.03
0.3 DON	0.89 ± 0.22	1.03 ± 0.16	1.15 ± 0.14	1.18 ± 0.18	0.76 ± 0.14
One-way ANOVA					
p value	.150	.044	.110	.183	.50

Note. Values are displayed as mean ± *SD*.

TABLE 5 Feed intake at different sampling time points for the long-term/low deoxynivalenol (DON) dosage experiment

	Day 37	Day 62	Day 92	Day 125	Day 168
Control	1.92 ± 0.06	1.67 ± 0.08	1.61 ± 0.07	1.43 ± 0.09	1.41 ± 0.03
0.3 DON	2.02 ± 0.08	1.85 ± 0.09	1.74 ± 0.10	1.59 ± 0.15	1.66 ± 0.68
One-way ANOVA					
p value	.133	.041	.109	.189	.070

Note. Values are displayed as mean ± *SD*.

(Figure 4). Vacuolation of hepatocytes was also more pronounced in 2.7 DON animals (5/10 fish) compared to the control animals (no registered cases of vacuolation of hepatocytes). In the 1.1 DON groups, hyalinized hepatocytes were visible (6/10 fish), but to a lesser extent compared with the 2.7 DON groups (8/10 fish). No significant differences were obvious between any of the experimental groups based on counts of the mucous cell numbers in the intestinal mucosa, pigmented macrophage centers in the liver and kidney, and number of necrotic single cells in the liver. No histological alterations were found in the intestine or kidneys (head and trunk kidney).

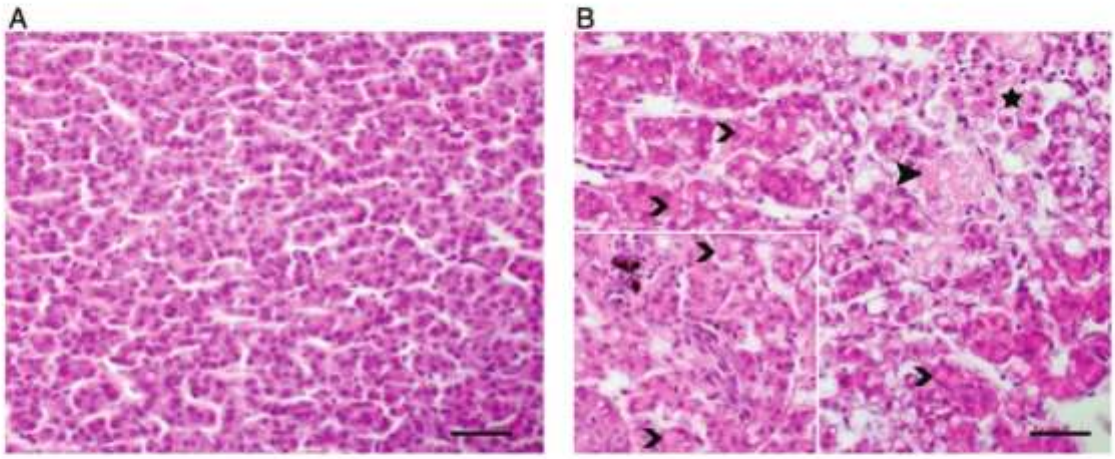

FIGURE 4 *Oncorhynchus mykiss*, histologic appearance of control (A) and 2.7 deoxynivalenol exposed fish (B); (A) normal structure of hepatocytes; (B) normal structure is disrupted, multiple hepatocytes are necrotic (star; observed in 1/10 fish sampled), scattered fibrin exudation (closed arrowhead; observed in 6/10 fish sampled), multiple hepatocytes show intracytoplasmatic eosinophilic, amorphous material (hyalinized hepatocytes) (open arrowheads; observed in 8/10 fish sampled), hematoxylin and eosin stain, bars = 50 μm; inlet: higher magnification showing hyalinized hepatocytes (open arrowheads)

3.4 | Challenge test

Cumulative mortality after inoculation with *Y. ruckeri* is shown in Figure 5. The challenge trial lasted 17 days, and the 2.7 DON treatment showed a significantly higher survival rate ($p < .020$) compared to the control treatment. Controls exhibited 73.3% survival, while the 1.1 DON and 2.7 DON treatments had a survival rate of 86.7% and 93.3%, respectively. No statistically significant differences were found between the 1.1 DON and 2.7 DON treatments or between the 1.1 DON treatment and the controls. The cause of death was confirmed as *Y. ruckeri* on the basis of the clinical signs. Furthermore, bacteria were reisolated from the kidneys of infected fish. In each case, pure cultures were obtained, and the colonies displayed morphology consistent with *Y. ruckeri*. This was further confirmed by isolating the genomic DNA from selected colonies and performing PCR using the primers described by del Cerro et al. (2002). Fish that had recovered from the infection at the time of the challenge termination did not display any gross clinical signs. Similarly, noninfected fish did not display any signs of infection.

3.5 | Liver enzymes

3.5.1 | Short-term DON exposure

The effects of the dietary treatments on LDH, ALT, and AST activities in the serum are summarized in Table 6. Samples from the fish that received the dietary DON appeared to have a higher LDH activity, although these results were not statistically significant ($p = .078$). The 2.7 DON treatment showed a significant increase in ALT and AST activities (76.10 ± 9.88 IU/L; $p < .001$ and 876.50 ± 87.60 IU/L; $p < .001$, respectively) compared with the control (ALT = 14.20 ± 7.66 IU/L and AST = 389.70 ± 2.36 IU/L; Table 6).

3.5.2 | Long-term DON exposure

Blood enzyme parameters measured at different sampling points are shown in Table 7. No significant differences were found during the experimental period for the different enzymes sampled.

3.6 | Clinical signs

Few clinical signs were observed in the fish exposed to the mycotoxin, and when abnormalities were observed, only a small number of fish were affected. Among the abnormalities were abnormal body conformations, observed in

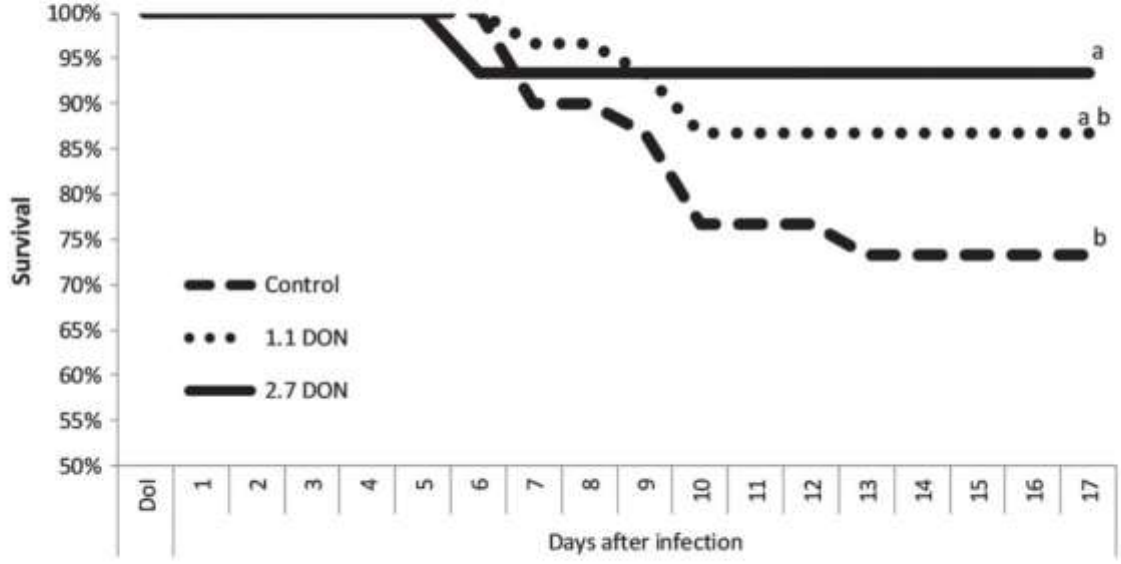

FIGURE 5 Survival curve following infection with *Yersinia ruckeri* during the high-dose experiment

TABLE 6 Effects of dietary treatments on lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate aminotransferase (AST) activities in the serum for short-term/high deoxynivalenol (DON) exposure experiment

	LDH (IU/L)	ALT (IU/L)	AST (IU/L)
Control	1,000.60 ± 187.01[a]	14.20 ± 7.66[a]	389.70 ± 2.36[a]
1.1 DON	2001.18 ± 825.06[a]	22.00 ± 0.97[a]	543.80 ± 45.68[a]
2.7 DON	1,700.60 ± 163.27[a]	76.10 ± 9.88[b]	876.50 ± 87.60[b]
One-way ANOVA			
p value	.078	<.001	<.001

Note. Data are presented as mean ± *SD*. Values in the same column with different letters are significantly different ($p < .05$).

TABLE 7 Effects of dietary treatments on lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (T-Prot), and hematocrit in the serum at different sampling time points for long-term/low deoxynivalenol exposure experiment

	Sampling	Hematocrit (%)	ALT (IU/L)	AST (IU/L)	LDH (IU/L)	ALP (IU/L)	T-Prot (g/L)
	Initial	51.2 ± 0.08	17.2 ± 11.8	432.9 ± 157.2	1846.5 ± 1,178.2	*	*
Control	62 days	39.9 ± 3.32	11.1 ± 3.5	309.1 ± 239.6	1862.7 ± 1,199.4	143.4 ± 71.8	3.0 ± 0.67
Mycotoxins		37.6 ± 4.29	24.4 ± 25.4	385.2 ± 91.55	2,497.0 ± 1,573.1	171.6 ± 69.5	3.0 ±0.5
Control	125 days	*	*	324.7 ± 144.4	1968.7 ± 1,222.8	154.4 ± 47.72	3.4 ± 0.79
Mycotoxins		*	*	216.5 ± 97.3	914.8 ± 314.9	146.3 ± 69.11	3.1 ± 0.63

Note. Asterisks denote values could not be determined due to technical problems with samples. Values are displayed as averages ± *SD*. $n = 5$ per treatment.

15 out of 60 fish that were fed 2,745 ± 330 μg/kg DON, characterized by a reduction in fish length in relation to width (Figure 6). In addition, in 5 out of 60 fish that were fed 2,745 ± 330 μg/kg DON, a protruding anal papilla was observed (Figure 7). Intussusceptions were observed internally in two fish.

4 | DISCUSSION

The decreasing supply and rising cost of fishmeal have led the aquaculture industry to investigate alternative sources of protein to substitute fishmeal in aquafeeds. Plant-based meals seem to be one of the most promising solutions for replacing fishmeal, and numerous plant raw materials have been successfully tested (Gatlin et al., 2007). However, recent studies have noted the occurrence of mycotoxins in plant-based aquafeeds (Barbosa et al., 2013; Gonçalves et al., 2016; Gonçalves et al., 2017; Greco et al., 2015; Nácher-Mestre et al., 2015; Pietsch, Kersten, Burkhardt-Holm,

FIGURE 6 Abnormal body conformations, characterized by a fish length reduced in relation to its width. Observed in 15 fish out of 60 fish fed 2,745 ±330 μg/kg deoxynivalenol

FIGURE 7 Fish presenting protruding anal papilla after being fed 2,745 ± 330 μg/kg deoxynivalenol (DON). Observed in 5 fish out of 60 fish fed 2,745 ± 330 μg/kg DON

Valenta, & Dänicke, 2013). In the present study, the experimental diets were contaminated with several mycotoxins and fungal metabolites in addition to the added DON. The presence of other mycotoxins and fungal metabolites highlights the risk of mycotoxin contamination in aquaculture finished feeds. The present experimental diet represents a typical commercial trout diet that contains plant-based compounds (59.70% DM). The co-occurrence of mycotoxins and fungal metabolites in this diet, even at low concentrations, may lead to synergistic/additive/antagonistic effects between these compounds, which cannot be ruled out as a contributing factor for the obtained results. However, further studies are needed to address possible interactions between mycotoxins, especially at low contamination levels.

The objective of the present trial was to evaluate the possible effects of DON contamination in aquaculture feeds under two different scenarios. In the first scenario, the effect of short-term feeding of high levels of DON (50 days; 1,166 μg/kg DON and 2,745 μg/kg DON) was examined in an attempt to mimic the potential inclusion of highly contaminated raw material(s) in the finished feed. This situation would normally affect only a few batches of feed; therefore, the exposure would occur over a short period. In this scenario, the potential influence of mycotoxins on *Y. ruckeri* susceptibility was also evaluated. The second experiment studied the effects of long-term exposure to low levels of DON (168 days; 367 μg/kg DON). This experiment was designed to replicate a situation that is more commonly found because 367 μg/kg DON is comparable to the average DON contamination level previously found in aquafeeds during recent years (Gonçalves, Hofstetter, et al., 2018; Gonçalves et al., 2016; Gonçalves et al., 2017).

One of the main constraints when researching mycotoxins in aquaculture species is the lack of mycotoxin-induced clinical symptoms. Although it is true that several published reports describe some clinical signs for the most common mycotoxins (see the review conducted by Anater et al., 2016), most of these clinical signs are very general and can be attributed to any other pathology or challenge faced by the animals, for example, antinutrition factors or lectins in the diet (Hart, Bharadwaj, & Brown, 2010). Furthermore, the clinical signs typically present high variability.

In this study, the occurrence of clinical signs was evaluated in both the short- and long-term exposure experiments, and special attention was paid to visual clinical signs. In the short-term/high DON exposure experiment, 15 out of 60 fish that were fed 2,745 ± 330 μg/kg DON showed an abnormal body conformation, characterized by a fish length reduced in relation to its width, and 5 out of 60 fish from same treatment presented a protruding anal papilla. No clinical signs were observed after long-term exposure/low DON exposure. Although clinical manifestation was observed in a small number of individuals (only at the higher dosage of the short-term/high DON exposure experiment), it cannot be concluded that the signs observed are directly attributed to DON. The rectal prolapse observed in some fish is also described as a DON clinical manifestation in swine when fed 3,000 μg/kg DON (Madson, Ensley, Patience, Gauger, & Main, 2014). However, a recent study (Gonçalves, Navarro-Guillén, et al., 2018) stated that no macroscopic lesions were found (i.e., internal or external hemorrhages, dermal and oral lesions, abnormal pigmentation, or damage to fins) on rainbow trout that were fed high levels of DON (11,412 ± 1,141 μg/

kg). Taking into account the previous study (Gonçalves, Navarro-Guillén, et al., 2018) and three other studies with the same range of DON contamination (0.3–5.9 ppm), Hooft et al. (2011) and Ryerse et al. (2015) also reported no major pathological changes in the distal intestine of trout, while Matejova et al. (2014) found gastrointestinal hemorrhages. It is possible that the impact of DON might vary greatly depending on unknown factors, even for the same species.

Recently, Gonçalves, Navarro-Guillén, et al. (2018) reported a novel DON metabolite (DON-3-sulfate) found in rainbow trout feces. The authors suggested that this biotransformation achieved by sulfation is probably realized by the trout gut microbiota as was previously described for other fish species (*Ameiurus nebulosus*; Guan et al., 2009). This biotransformation, if achieved by the gut microbiota, can also help to explain the high individual variability obtained, as the capacity to metabolize DON will be directly influenced by the individual fish microbiome. This explains the absence of clinical signs in some of the fish that were fed DON because DON-3-sulfate is less toxic than DON. The high interindividual variation within the groups that were fed mycotoxins highlights the importance of the individual health and nutritional status prior to DON ingestion, as supported by other authors (Hendricks, 1994). Due to the reasons previously stated, the clinical manifestation found in the present study, even if only present in a small number of individuals, should be further confirmed as a DON-exclusive clinical sign, associating it with an individual fish microbiome.

Reduction in FI is a well-documented response of rainbow trout to diets contaminated with naturally occurring or artificially added DON (Gonçalves, Navarro-Guillén, et al., 2018; Hooft et al., 2011; Ryerse et al., 2015). In the present short-term study, fish that were fed 2,745 μg/kg of DON showed a significant reduction ($p < .001$) in FI. However, no effect was observed in fish that were fed 1,166 μg/kg of DON. A significant decrease in growth was also detected in the 2.7 DON treatment; TGC decreased by 17% ($p = .001$), and SGR decreased by 13% ($p < .001$). However, no significant differences ($p > .05$) were found for PER or FCR. In the long-term study, ingestion of DON was asymptomatic, as the animals presented no clinical signs, and growth rate was slightly affected only after 92 days of ingesting DON. At the end of the trial (168 days), the animals that were fed DON weighed less than the control animals. Although not significantly different, the tendency for reduced weight gain in animals that were fed DON is consistent with the short-term experiment. Recently, Gonçalves, Navarro-Guillén, et al. (2018) suggested that suppression of appetite due to DON contamination in feeds might be a defense mechanism to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The authors showed that pituitary adenylate cyclase-activating polypeptide seems to be fundamental for explaining the reduction of FI in DON-fed treatments, inducing anorexia, reinforcing the influence of DON on the hypothalamic melanocortin system. It is also important to mention that a contamination dose of 367 μg/kg of DON is a frequent and plausible level of contamination that is often found in aquafeeds incorporating plant meals (Gonçalves et al., 2016; Gonçalves et al., 2017). Moreover, this value is close to the limit of detection of most commercial ELISA (enzyme-linked immunosorbent assay) strip tests for DON, which means that samples need to be analyzed by more robust methods (e.g., HPLC), which increases costs and the time to receive sample results. The observed asymptomatic decrease in growth performance may lead to important economic consequences for the aquaculture industry.

In both experiments, it was difficult to correctly diagnose DON intake using the other parameters evaluated (liver enzymes and histology). In the short-term/high DON exposure study, histological and enzymatic changes showed different results, and individual variability was very high. Enzymatic activity was used to evaluate the possibility of tissue destruction. ALT and AST have previously been used as markers of liver dysfunction (Gül, Belge-Kurutaş, Yildiz, Sahan, & Doran, 2004; Saravanan, Usha Devi, Malarvizhi, & Ramesh, 2012), and ALT is an intracellular enzyme that has been used as a marker of tissue destruction in the liver. However, no clear pattern could be observed in the studies. Only in the short-term/high DON exposure study were elevated ALT serum levels found in the 2.7 DON treatment compared with the control group. In addition, AST values were significantly higher in the 2.7 DON treatment compared with the control. Elevated ALT and AST serum levels might be an indication of liver or other parenchymal organ damage. Liver histopathology revealed mild to moderate damage in

a limited number of DON-exposed fish. However, no histological alterations were detected in the intestine or kidneys (head and trunk kidney). DON is known to cause impairment of barrier integrity, affecting the lamina propria and tight junctions, which may increase gastrointestinal tract (GIT) permeability and consequently allow the entry of luminal antigens and bacteria normally restricted to the GIT lumen (Dänicke, Valenta, & Döll, 2004; Grenier & Applegate, 2013). The fact that histological alterations were not found in the intestines, despite the altered values of ALT and AST, might lead us to hypothesize that short exposure periods might not be sufficient to lead to histological alterations and/or that histology might not be a good method to evaluate negative DON effects in the intestines. Moreover, as mentioned by Gonçalves, Navarro-Guillén, et al. (2018), the individual microbiome seems to play an important role in DON biotransformation, which may also influence the obtained histological results. It would also be interesting to more closely examine the tight junction proteins as a more sensitive indicator for possible DON impact at the intestinal barrier, specifically at the tight junction level.

The results obtained for the *Y. ruckeri* challenge are consistent with the results from previous studies that investigated the effect of dietary DON on the mortality of rainbow trout challenged with other bacterial pathogens (Hooft et al., 2011; Ryerse et al., 2015). The apparent absence of an immunosuppressive effect on trout challenged with DON contrasts with published data for livestock species such as swine (Lessard et al., 2015; Pierron, Alassane-Kpembi, & Oswald, 2016). An eventual direct suppression of *Y. ruckeri* by DON seems unlikely as it is very well described that trichothecenes interact with the eukaryotic 60S ribosomal subunit and prevent polypeptide chain initiation or elongation (Carter & Cannon, 1977; Pestka, 2007; Ueno, 1984). The present study did not include a pair-fed group (i.e., a group consuming the same amount of feed as that consumed by the DON groups), and thus it was not possible to distinguish the effects of feed restriction (caused by DON) from other effects of DON that might have decreased susceptibility to *Y. ruckeri*.

The intake of DON has been reported to lead to the upregulation of cytokine levels, especially proinflammatory cytokines (IL-6, IL-8, and IL-1β), in several studies (piglets, Bracarense et al., 2012; human intestinal Caco-2 cells, Maresca et al., 2008, Van De Walle, Romier, Larondelle, & Schneider, 2008; and mice, Azcona-Olivera, Ouyang, Murtha, Chu, & Pestka, 1995). Intestinal upregulation of proinflammatory cytokines may explain the higher resistance of DON-treated fish to infection with *Y. ruckeri*. However, as explained by Grenier and Applegate (2013), DON, as a protein synthesis inhibitor, might naturally originate superinduction phenomena, consequently increasing cytokine synthesis and secretion. Nonetheless, the possible role of DON in the upregulation of proinflammatory cytokines and the consequent effect on immune stimulation should be further investigated.

5 | CONCLUSION

The present findings reinforce those from previous studies, concluding that the ingestion of DON by trout over short-term periods at high dosages (50 days; 1,166 μg/kg and 2,745 μg/kg) impacts growth performance, especially FI, with minor or variable biochemical changes in trout blood and histopathological changes. In this case, some fish did exhibit clinical symptoms (i.e., anal papilla), which could be attributed to the DON treatment; however, further confirmation is needed. This is the first report of the effects of the long-term exposure of rainbow trout to low concentrations of DON (168 days; 367 μg/kg DON). Ingestion of DON in the long-term study was asymptomatic; however, the fish started to reduce their growth performance 92 days after ingesting DON. Such low contamination levels, which might be unnoticed by farmers, may have economic consequences for aquaculture.

DON-treated fish showed higher resistance to infection with *Y. ruckeri*, which may be related to stimulation of the proinflammatory response. While higher resistance to pathogen infection may be considered as a positive effect,

the reduced FI and lower growth performance may have economic consequences for aquaculture. Moreover, further investigation is needed to understand the influence of DON on proinflammatory responses.

The high levels of individual variability observed in the blood biochemical parameters, histological changes, and clinical signs in the fish that were fed DON might be explained by individual intestinal microbiota composition. The individual gut microbiome and its apparent capacity to metabolize DON should be further explored.

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• CHAPTER 3 - Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism was based on:

Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism

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Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism

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ABSTRACT

The impact of deoxynivalenol (DON) on rainbow trout, *Oncorhynchus mykiss*, is mainly characterised by impaired growth performance and reduced feed intake, usually with the total absence of any visible clinical signs. Despite the high concentrations of DON in the present study (up to 11,412 ± 1141 μg kg^{-1}), no clinical signs (except anorexia at the higher DON dosage) were observed, which confirms the difficulties of diagnosing DON ingestion. Compared to the control group, the proteolytic enzyme activities (pepsin, trypsin and chymotrypsin) in trout were altered by DON ingestion. However, it was not clear if the observed impact on digestive enzymes was due to the direct action of DON, or a consequence of the lower feed intake determined for DON-treated animals. The impact of DON on the abundance of specific measured mRNA transcripts was unexpected with higher expression levels for insulin-like growth factors, *igf1* and *igf2*, which are directly related to elevated insulin levels in plasma. This can also in part be influenced by the trypsin activity and by *npy*, given its higher mRNA expression levels. The apparent digestibility of dry matter, protein and energy was not affected by dietary levels of DON, however, nutrient retention, protein, fat and energy retention were significantly affected in animals fed DON. Adenylate cyclase-activating polypeptide (PACAP) expression seems to play an important role in controlling feed intake in DON fed trout. In the present study, we have shown for the first time that DON is metabolized to DON-3-sulfate in trout. DON-3-sulfate is much less toxic than DON, which helps to explain the lack of clinical signs in fish fed DON. Being a novel metabolite identified in trout makes it a potential biomarker of DON exposure. Suppression of appetite due to DON contamination in feeds might be a defense mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON.

1. Introduction

Mycotoxins, toxic secondary metabolites produced by moulds (Hussein and Brasel, 2001), can be produced on agricultural commodities before and/or after harvest, during transportation or storage. Mycotoxins are a significant problem worldwide causing adverse health outcomes when consumed by humans and animals (Zain, 2011) and are responsible for significant economic losses worldwide due to condemned agricultural products (CAST, 2003; Shane and Eaton, 1994; Vasanthi and Bhat, 1998). The presence of mycotoxins in aquaculture are not novel. The first report of mortality due to mycotoxicosis in aquaculture was in the early 1960s, where in the United States, hatchery-reared rainbow trout (*Oncorhynchus mykiss*) were fed cottonseed meal contaminated with aflatoxins (Kumar et al., 2013; Wolf and Jackson, 1963).

In recent years, the awareness of mycotoxin-related issues in the industry has been raised again, mainly due to the increasing inclusion levels of plant meals in aquafeeds (Anater et al., 2016; Gonçalves et al.,

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Abbreviations[1]

Gene abbreviations

ef1a	elongation factor 1 alpha
actb	beta actin
star	steroidogenic acute regulatory protein
Igf1	insulin-like growth factor 1
Igf2	insulin-like growth factor 2
crf1	corticotropin releasing factor precursor 1
crf2	corticotropin releasing factor precursor 2
crfbp	corticotropin releasing factor binding protein precursor
npy	neuropeptide Y precursor
adcyap1a	growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide (PACAP)
lep	leptin
sst2	somatostatin-2 precursor
chia	gastric chitinase
pga	pepsinogen
lpl	lipoprotein lipase
ghrl	ghrelin/obestatin preprohormone
cel1	carboxyl ester lipase 1
cel2	carboxyl ester lipase 2
cckt	cholecystokinin (Tyrosine)
cckn	cholecystokinin (Asparagine)
cckl	cholecystokinin (Leucine)
amy2a1	pancreatic alpha amylase
atp4a	ATPase H+/K+ transporting alpha subunit
crtl	chymotrypsinogen-like precursor
try1	trypsinogen 1 precursor
try2	trypsinogen 2 precursor
try3	trypsinogen 3 precursor

Mycotoxins abbreviations

AF	aflatoxin
DON	deoxynivalenol
DOM-1	deepoxy-deoxynivalenol
DON-3-sulfate	deoxynivalenol-3-sulfate
DOM-3-sulfate	deepoxy-deoxynivalenol-3-sulfate
FUM	fumonisin
OTA	ochratoxin A
ZEA	zearalenone

Other abbreviations

ADC	apparent digestibility coefficients
GIT	gastrointestinal tract
ABW	average body weight

2016, 2017; Hooft and Bureau, 2017; Hooft et al., 2011). Feed manufacturers and producers realise the importance of mycotoxins and their potential negative effects on production. However, there are still two major constraints entrenched in the aquaculture industry that make it difficult to develop an effective mycotoxin management plan for the sector.

The first constraint is the perception that the majority of mycotoxin issues stem from poor storage conditions. Poor storage conditions can lead to the growth of *Aspergillus* spp. and *Penicillium* spp., which can ultimately lead to the production of aflatoxin (AF) and ochratoxin A (OTA). In countries where climate conditions are favourable to the growth of *Aspergillus* spp. and *Penicillium* spp. fungi, optimal storage conditions should prevent the contamination of raw materials and finished feeds with AF or OTA. However, the same is not true for *Fusarium* spp., which on the contrary to *Aspergillus* spp. and *Penicillium* spp., are more likely to grow in the crops pre-harvest. The *Fusarium* mycotoxins (Type B and A trichothecenes and fumonisins) are reasonably stable to processing conditions (Cheli et al., 2013). Therefore, these mycotoxins are not destroyed during raw material processing or aquafeed manufacturing, and will consequently be present in the finished feed. This may lead to potential harm to fish and shrimps, dependent upon concentration and co-occurrence. We have previously reported that soybean meal, wheat, wheat bran, maize, corn gluten meal, rapeseed/canola meal and rice bran in samples from Asia were mostly contaminated with *Fusarium* mycotoxins (zearalenone, ZEA; deoxynivalenol, DON; and fumonisin B_1, FB_1) (Gonçalves et al., 2016, 2017). Thus, finished feed samples were mainly contaminated with *Fusarium* mycotoxins, reflecting the use of plant meals in the finished feeds and not resulting from poor storage conditions (Gonçalves et al., 2016, 2017). Moreover, the presence of secondary metabolites of *Fusarium* spp. are expected to increase as a response to climate change (Miraglia et al., 2009; Paterson and Lima, 2010, 2011), which will likely further impact the global aquaculture industry. Among the metabolites produced by the *Fusarium* genus, DON is reported as the main mycotoxin found especially in small grain cereals (Rodrigues and Naehrer, 2012; Simsek et al., 2013). Despite the fact that the toxic effects and toxicokinetics of DON - a mycotoxin commonly known as "vomitoxin" as it causes vomiting in livestock - is well described including clinical symptoms for land farmed animals (Pestka, 2007), very little is known for aquatic animals.

The second constraint for mycotoxin research in aquaculture is the lack of any validated clinical symptoms in fish and shrimps when they are fed mycotoxins. In the case of DON, no known distinct subclinical signs of DON toxicoses in fish (i.e. no distinct lesions/pathologies) are described for aquaculture species. Several reports describe some clinical signs for the most common mycotoxins (see review from Anater et al. (2016)), however, they are generalised and could be attributed to any diverse pathologies or challenges e.g. anti-nutrition factors or lectins in the diet (Hart et al., 2010). Two notable exceptions are aflatoxicosis (yellowing of the body surface, (Deng et al., 2010) and ingestion of FB (alteration of the sphinganine to sphingosine ratio, (Tuan et al., 2003)). Most reported clinical manifestations due to mycotoxin ingestion are related to a reduction in growth performance, alteration of blood parameters (erythrocyte/leucocyte count), blood enzymes (Alanine Aminotransferase (ALT), Aspartate Transaminase (AST) or Alkaline Phosphatase (ALP)), liver alterations or the suppression of immune parameters. Moreover, it is generally observed that the biological effects of mycotoxins vary greatly over different species. Even in the same species, they depend on the concentration of the toxin in feed, the age of the animal, and its nutritional and health status prior to mycotoxin ingestion (Hendricks, 1994).

The present work aims to evaluate and elucidate the impact of DON on rainbow trout, by exploring new tools and evaluating new diagnostic factors, which may be used later by the industry as standards to better diagnose mycotoxicoses in fish. Reduced growth performance is one of the most prevalent clinical signs of DON across fish species. Building upon the current knowledge of the impact of DON in rainbow trout (Hooft and Bureau, 2017; Hooft et al., 2011; Matejova et al., 2015; Ryerse et al., 2015), we have attempted to characterise reduced growth performance by exploring the impact of DON on ingredient digestibility. This was achieved by measuring total pepsin, trypsin, chymotrypsin, amylase and lipase activities. The expression level of gene

[1] ZFIN Zebrafish Nomenclature Guidelines have been followed for all fish genes and proteins described in this manuscript (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines#ZFINZebrafishNomenclatureGuidelines-1).

markers for stress regulation were assessed at head kidney (*star*) and brain (*crf1*, *crf2*, *crfbp*). For growth control, liver was evaluated for *igf1 and igf2*, and brain for *adcyap1a* (*PACAP*). Regarding digestion regulation and appetite control was evaluated expression of *sst2*, *chia*, *pga*, *lpl*, *ghrl*, *cel1*, *cel2*, *cckt*, *cckn*, *cckl*, *amy2a1*, *atp4a*, *crtl*, *try1*, *try2* and *try3* on gastrointestinal tract (GIT) and *lep and npy* in brain. Moreover, due to DON-related damage of the GIT, faeces was analysed for DON metabolites in order to study the DON metabolism.

2. Material and methods

2.1. Experimental diets

The trial comprised three dietary treatments (Table 1), all based on a single dietary formulation. The control diet (CTRL) contained a commercially relevant level of fishmeal (Fishmeal Super Prime = 12.45%; Fishmeal 60 = 14.0%) and moderate levels of plant ingredients such as soy protein concentrate, wheat gluten, corn gluten, wheat meal, soybean meal and corn meal as protein sources. Fish oil was used as the main lipid source. This control diet served also as the base of two additional diets which were supplemented, at the mixing step, with culture material extract containing DON (Romer Labs, Tulln, Austria) at 4.5 and 10.5 mg kg^{-1} (diets DON 5 and DON 11, respectively). Contamination levels were chosen taking into account previous literature (Hooft et al., 2011; Matejova et al., 2014, 2015; Ryerse et al., 2015) instead of reported DON levels in European finished feed samples (Gonçalves et al., 2016). This because, reports of mycotoxin occurrence in European aquaculture finished feeds are still very limited, and levels reported (average DON contamination of 165.61 µg kg^{-1} Gonçalves et al., 2016) might be underestimated. All diets were isonitrogenous (crude protein, 52.2% dry matter (DM)), isolipidic (17.9% DM) and isoenergetic (gross energy, 22.2 MJ kg^{-1} DM).

2.2. Manufacture of diets

Diets were manufactured by extrusion (pellet size = 2.0 mm) at SPAROS (Portugal) using a pilot-scale BC45 twin-screw extruder (CLEXTRAL, France) with a screw diameter of 55.5 mm; the operating temperature was 113–116 °C. Upon extrusion, feeds were dried in a vibrating fluid DR100 bed dryer (TGC Extrusion, France). Pellets were allowed to cool to room temperature before application of the oil fraction by coating under vacuum conditions (PG-10VCLAB instrument; DINNISEN, The Netherlands). The target amount of oil for the post-extrusion coating procedure was sprayed onto the pellets under vacuum (760 mbar) for approximately 2 min. The experimental feeds were stored in a cool and aerated location throughout the trial. Samples of each diet were taken for proximate composition analysis (Table 1). A sample of each diet was tested for target mycotoxin presence (DON; Table 1) plus other relevant mycotoxins and metabolites (Table S1). The natural presence of other major mycotoxins, were determined analytically as described previously and reported in table S2 (Streit et al., 2013).

2.3. Fish and rearing conditions

All procedures involving fish were performed according to the EU guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Quadruplicate groups of 50 rainbow trout (*Oncorhynchus mykiss*), with a mean ± standard deviation (s.d.) initial body mass (IBM) of 2.52 ± 0.03 g, were fed one of the three experimental diets for 60 days. Fish were grown in quadrangular flat-bottom fish tanks (V = 250 L) supplied with well freshwater in a flow-through system; 14.3 ± 0.4 °C water temperature, 8.1 ± 0.7 mg L^{-1} dissolved oxygen, and a 14 h light: 10 h dark photoperiod regime. Fish were hand fed to visual satiety three times per day (twice during weekends) with utmost care to avoid feed wastage and allow a precise quantification of feed intake. Furthermore observing feeding behaviour allowed us to confirm that the reduced feed intake in DON fed treatments were established progressively after a certain time accepting the feeds, excluding the possibility that reduced food intake due to modified organoleptic properties of the feed. Fish were anesthetised with 2-phenoxyethanol (200 mg L^{-1}) for group weighing at the start of the trial (day 0), at day 29, and at day 60.

2.4. Biological sampling

The whole-body proximate composition was analysed from a pool of ten fish sampled and stored (−20 °C) at the beginning of the trial (day

Table 1
Formulation and composition of experimental diets.

Ingredients, %	CTRL	DON 5	DON 11
Fishmeal 60[a]	14.00	14.00	14.00
Fishmeal Super Prime[b]	12.45	12.45	12.45
Soy protein concentrate[c]	15.00	15.00	15.00
Wheat gluten[d]	12.30	12.30	12.30
Corn gluten meal[e]	8.00	8.00	8.00
Soybean meal[f]	6.00	6.00	6.00
Wheat meal[g]	6.40	6.40	6.40
Corn meal[h]	10.00	10.00	10.00
Fish oil[i]	10.00	10.00	10.00
Soy lecithin[j]	2.00	2.00	2.00
Antioxidant[k]	0.30	0.30	0.30
Monocalcium phosphate[l]	1.50	1.50	1.50
L-lysine[m]	0.50	0.50	0.50
DL-methionine[n]	0.50	0.50	0.50
Vitamin E[o]	0.05	0.05	0.05
Vitamin and mineral premix[p]	1.00	1.00	1.00
Deoxynivalenol (mg kg^{-1})	-	4.5	10.5
Proximal composition			
Dry matter (DM), %	91.7 ± 0.0	91.5 ± 0.0	91.6 ± 0.0
Crude protein, % DM	52.2 ± 0.1	52.2 ± 0.0	52.2 ± 0.0
Crude fat, % DM	17.9 ± 0.0	17.8 ± 0.0	17.8 ± 0.0
Ash, % DM	9.3 ± 0.0	9.3 ± 0.0	9.3 ± 0.0
Gross energy, kJ g^{-1} DM	22.2 ± 0.0	22.2 ± 0.0	22.2 ± 0.0
Chromium oxide, % DM	0.96 ± 0.02	0.96 ± 0.01	0.95 ± 0.01
Analysed mycotoxin concentrations (ppb = µg kg^{-1})			
Deoxynivalenol	37.42 ± 0.0	4714 ± 566	11,412 ± 1141
Zearalenone	78.63 ± 0.0	78.63 ± 0.0	78.63 ± 0.0
Fumonisin B_1	67.73 ± 0.0	67.73 ± 0.0	67.73 ± 0.0
Sum of ergot alkaloids	Not detected	Not detected	Not detected
Aflatoxin B_1	Not detected	Not detected	Not detected

[a] COFACO 60: 62.3% crude protein (CP), 8.4% crude fat (CF), COFACO, Portugal.
[b] Super Prime: 67.4% CP, 8.2% CF, EXALMAR, Peru.
[c] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[d] VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France.
[e] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[f] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain.
[g] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.
[h] Corn meal: 8.1% CP; 3.7% CF, Casa Lanchinha, Portugal.
[i] SAVINOR, Portugal.
[j] Lecico P700IPM, LECICO GmbH, Germany.
[k] Paramega PX, Kemin Europe NV, Belgium.
[l] MCP: 22% P, 18% Ca, Fosfitalia, Italy.
[m] Lysine HCl 99%, Ajinomoto Eurolysine SAS, France.
[n] DL-Methionine 99%, EVONIK DEGUSSA GmbH, Germany.
[o] ROVIMIX E50, DSM Nutritional Products, Switzerland.
[p] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

0) and from a pool of three fish per tank sampled and stored (−20 °C) at the end of the trial (day 60). Additionally, at the end of the growth trial, ten fish per tank were anesthetised with 2-phenoxyethanol (200 mg L^{-1}) and a blood sample was collected by puncturing the caudal vein with a heparinised syringe. Blood samples were centrifuged at 1590 × *g* for 10 min and the resulting supernatant fraction, i.e. the plasma, was transferred to a clean vial, snap-frozen in liquid nitrogen, and stored at −80 °C for the subsequent analysis of metabolites. Immediately after blood collection, fish were euthanized by anaesthetic overdose (prolonged exposure to 2-phenoxyethanol (200 mg L^{-1})) and their livers were dissected and weighed for calculating the hepatosomatic index (HSI). Moreover, samples of stomach, liver, pancreas and intestine (n = 10/treatment) were preserved in liquid nitrogen and stored at −80 °C for enzyme activity measurement. Samples of brain, GIT, liver and head kidney (n = 10/treatment) were flash frozen in liquid nitrogen, with the exception of brain samples which were preserved in RNAlater® (Invitrogen Life Technologies) for gene expression analysis. All samples were stored at −80 °C until processed. Sample collection was done randomly 3 h after the animals were fed.

2.5. Biochemical composition of feeds, whole fish, and faeces

Analyses of feed, whole fish, and faeces were carried out with analytical duplicates following the methods described by the Association of Official Analytical Chemists (AOAC, 2006). Dry matter was measured after drying at 105 °C for 24 h. Total ash was analysed by combustion (550 °C for 6 h) in an L9/11/B170 muffle furnace (NABERTHERM, Germany). Crude protein (N × 6.25) was analysed by flash combustion followed by gas chromatographic separation and thermal conductivity detection with a Leco N FP-528 analyser (LECO Corporation, MI, USA). Crude lipid was determined by petroleum ether extraction (40–60 °C) using a SOXTEC™ 2055 Fat Extraction System (Denmark). Gross energy was measured in an adiabatic C2000 basic bomb calorimeter (WERKE, Germany), and chromium concentration in feeds and faeces was determined by atomic absorption spectrometry in a SpectrAA 220 FS instrument (VARIAN, CA, USA) (Reis et al., 2008).

2.6. Mycotoxin analyses in feed

The analyses were carried out as described by Streit et al. (2013). All samples were analysed with liquid chromatography tandem mass spectrometry (LC-MS/MS). For the purpose of data analysis, non-detect levels were based on the limit of detection (LOD) of the LC-MS/MS. The LOD for aflatoxin B_1, was 0.3 μg kg^{-1}. For ZEA, DON and OTA, the LODs were 10, 50 and 0.2 μg kg^{-1}, respectively. For FUM the LOD was 25 μg kg^{-1} for FB_1, FB_2, FB_3, and FB_4. The LOD for the remaining toxins/metabolites was 0.5 μg kg^{-1}.

2.7. Apparent digestibility measurements and mycotoxin analysis in fish faeces

At the end of the growth performance trial, and following the sampling procedures described in Section 2.3, ten fish per tank were used to determine the apparent digestibility coefficients (ADC) of dry matter, protein, and energy using the indirect method. An inert tracer (0.96% Chromium oxide, Cr_2O_3) was added to the feed and the nutrient to tracer ratio in feed and faeces were used for digestibility measurements. Faeces samples were collected using the apparatus for continuous faeces collection by filtration described by Choubert Jr. et al. (1979). Over the course of one week, faeces was removed from filters 3 h after each feeding and stored at −20 °C. Faeces collected per tank was pooled per treatment and stored at −20 °C for subsequent analysis. Faeces was also analysed for the presence of DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate). Briefly, four 500 mg samples were extracted in duplicate three times from each treatment using 5 mL of 50% methanol. The samples were vortexed and shaken for 30, 20 and 10 min. The extraction samples were centrifuged – first and second centrifugations at 3200 rpm for 5 min, and the final centrifugation at 4200 rpm for 5 min - and the supernatants combined, vortexed and centrifuged again at 4200 rpm for 5 min. Prior to LC-MS/MS analysis, samples were diluted in vials (1:1) with the extraction solvent as described by Schwartz-Zimmermann et al. (2015).

2.8. Enzyme activity analyses

Enzyme extracts were prepared for enzyme activity measurement from samples previously preserved in liquid nitrogen and stored at −80 °C. Stomach and intestine samples were dissected and homogenised separately. Samples were manually homogenised in 3 mL distilled water and centrifuged for 10 min at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from the stomach samples were measured for pepsin activity, and the supernatants from the intestine samples were analysed for trypsin, chymotrypsin, amylase and lipase activities.

Pepsin activity was determined by the method of Anson (1938): 15 μL of extracts were mixed with 1 mL of 0.5% acid-denatured bovine hemoglobin diluted in 0.2 M HCl-Glycine buffer (pH 3). After incubation at 37 °C for 30 min, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C for 15 min and then centrifuged at 12,000 rpm for 15 min. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA. For alkaline protease activities, trypsin activity was assayed using BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide) (B4875 Sigma-Aldrich) as a substrate. 0.5 mM BAPNA was dissolved in 1 mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50 mM, pH 8.5, containing 20 mM $CaCl_2$. Chymotrypsin activity was determined using 0.2 mM SAPNA (*N*-succinyl-L-Ala-L-Pro-Phe-*p*-nitroanilide) dissolved in 1 mL DMSO and then made up to 100 mL in the same buffer. Reactions were started in 96-well microplates by the addition of 15 μL of the enzyme extract to 200 μL of the respective substrate and liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA).

Lipase activity was measured following the method described by Versaw et al. (1989), with some modifications. The assay mixture contained 60 μL of 100 mM sodium taurocholate, 540 μL of 50 mM Tris-HCl, pH 8.5, 10 μL of enzyme extract and 6 μL of β-Naphthyl caprilate. The reaction was maintained for 25 min at 37 °C and after this time, 6 μL of 100 mM Fast Blue BB in DMSO was added before being incubated at 37 °C for 5 min. The reaction was then stopped with 60 μL TCA 0.72 N. Finally, 815 μL of 1:1 (v:v) ethyl acetate/ethanol solution was added and the absorbance recorded at 540 nm.

Amylase activity was determined by the 3,5-dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 2% soluble starch as a substrate. 30 μL of enzyme extract and 300 μL of substrate were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 150 μL DNS and was heated in boiling water for 10 min. Then, after cooling in ice, 1.5 mL of distilled water was added to the mixture and the absorbance was measured at 540 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after DNS.

2.9. Gene expression quantification

All the samples were individually processed for total RNA extraction using NucleoSpin® RNA kits (Macherey Nagel). An Ultra-Turrax® T25 with an S25N-8G dispersion tool (IKA®-Werke) was used to homogenise the brain tissue in a volume of homogenising buffer proportional to the mass of the tissue. The remaining samples (initially frozen in liquid nitrogen) were homogenised in three steps. First, using a mortar and a pestle, then with an Ultra-Turrax® T25 in liquid nitrogen and finally by taking a sample of < 30 mg from the finely minced powder to process with an Ultra-Turrax® T10 with the kit homogenising buffer. Genomic

DNA (gDNA) was removed via on-column DNase digestion at 37 °C for 30 min using rDNase (RNase-free) included in the kit. The RNA concentration was measured with a Qubit 2.0 fluorimeter and Qubit RNA BR assay kit (Life Technologies), whereas RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit (Agilent Technologies). Only samples with an RNA integrity number (RIN) > 8.0 were tested using real-time quantitative PCR (qPCR). Total RNA (500 ng) from each sample was reverse-transcribed in a 20 μL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences) in a Mastercycler® proS (Eppendorf) and as previously described by Mata-Sotres et al. (2016). A pool of cDNA from all the samples for each tissue was used for calibration plots, using six serial 1/10th dilutions from 10 ng to 100 fg, in order to assess the linearity and efficiency of the different primer combinations, as well as for being used for inter-assay calibration. Control reactions with RNase free water (NTC) and RNA (NRT) were included in the analysis to ensure the absence of primer-dimers and genomic DNA contaminations. The linearity and amplification efficiency for each pair of primers are shown in Table S1. Previously, primers pairs were tested for final working concentrations (optimum 200 nM) and temperature (60 °C). qPCR reactions were performed in triplicate with 1 ng of cDNA (estimated from the input of total RNA) forward and reverse primers (Table S1) for the named samples (200 nM each) and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences). Reactions were performed in a volume of 10 μL using Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). The thermocycling procedures were carried out with an initial denaturation and polymerase activation at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing and extension at 60 °C for 30 s, and finishing with a melting curve from 60 °C to 95 °C, increasing by 0.5 °C every 5 s. Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer–dimer artifacts. Relative gene expressions were quantified in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalised by geometric averaging of two references genes (Vandesompele et al., 2002), *actb* and *ef1a*. The genes were selected owing to their lower than 0.5 target stability M value and lower than 0.25 CVs (as indicated by BioRad CFX Manager Target Stability Value). A pool of all the cDNA samples was used as a calibrator on every qPCR plate to correct for inter-assay differences. qPCR reactions were carried out with 10 ng of cDNA (assumed from RNA input), forward and reverse primers, and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences) in a final volume of 10 μL. qPCR primer sequences, amplicon sizes, amplification efficiencies, R^2 and GenBank accession number of the sequences are shown in Table S1. RNA nucleotide sequences for *ef1a*, *actb*, *star*, *igf1*, *igf2*, *crf1*, *crf2*, *crfbp*, *npy*, *adcyap1a*, *lep*, *sst2*, *chia*, *pga*, *lpl* and *ghrl*, were obtained from GenBank, and nucleotide sequences for *cel*, *cel2*, *amy2a*, *atp4a*, *crtl*, *try1*, *try2* and *try3* were retrieved from the *Oncorhynchus mykiss* WGS project database, using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer/).

2.10. Growth, feed intake, digestibility, and nutrient budget calculations

IBW (g): Initial mean body weight

FBW (g): Final mean body weight

Specific growth rate (SGR; %/day) = (Ln FBW − Ln IBW) × 100/days

Feed conversion ratio (FCR) = crude feed intake / weight gain

Feed intake (FI; %BW/day) = (crude FI (DM feed) / (IBW + FBW) / 2/days) × 100

Protein efficiency ratio (PER) = wet weight gain / crude protein intake

Hepatosomatic index (HSI) = (liver weight / body weight) × 100

Nutrient retention (%) = ((FBW × NFF) − (IBW × NIF)) / Nutrient intake × 100, with NFF being the nutrient content of final fish and NIF the nutrient content of initial fish.

Apparent digestibility coefficient (ADC, %) = 100 × [1 − (% Cr_2O_3 feed/Cr_2O_3 faeces) × (% nutrient faeces / % nutrient feed)]

Daily average nitrogen (N) gain = ((final body N content − initial body N content) / (IBW + FBW))/2/days

Daily N intake = (N intake / (IBW + FBW)/2)/days

Daily faecal N losses = daily N intake × (100 − ADC Protein) / 100

Daily metabolic N losses = daily N intake − (daily N gain + daily faecal N losses)

2.11. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare differences between the three diets. A post-hoc Tukey honest significant difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The Shapiro-Wilk test was used to analyse the normality and homogeneity of variances was tested using Levene's test. Data analysed did not violate the assumption of equal variances and showed normal distribution. All parameters expressed as percentages were subjected to arcsin square root transformation. Data are presented as the mean of quadruplicates ± standard deviation. All statistical tests were performed in IBM SPSS Statistics 18 software (IBM Corp., USA).

3. Results

3.1. Zootechnical performance

3.1.1. After 29 days of experimental feeding

The survival rate of the rainbow trout was high (> 97.0%), across the three dietary treatments with only minor mortality in the DON 5 and DON 11 treatments, that was not significant ($P > 0.05$; Table 2). After 29 days, all other zootechnical parameters (FBW, g; SGR, %/d; FCR; FI, %; ABW/d; PER), were significantly affected by dietary inclusion of DON ($P < 0.001$; Table 2). FBW ranged from 3.90 to 9.39 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR, FI and PER than those fed both DON supplemented diets (DON 5 and DON 11) ($P < 0.001$). Additionally, fish fed the highest DON dose (DON 11) showed a significantly lower FBW, SGR, FI and PER than those fed with the DON 5 diet ($P < 0.001$). The FCR varied between 0.96 and 1.56. Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet ($P < 0.001$).

3.1.2. After 60 days of experimental feeding

After the second experimental period, mortality was observed in all treatments (Table 3). The survival rate of the fish fed CTRL and DON 5 diets was significantly higher than that observed in fish fed the DON 11 diet ($P = 0.02$). Fish from the best performing treatment (CTRL) had a 9.8-fold increase of their initial body mass, with FBW ranging from 5.96 to 24.77 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR and FI than those fed both DON supplemented diets (DON 5 and DON 11; $P < 0.001$). Additionally, fish fed the highest DON dose (DON

Table 2
Growth performance after 29 days of feeding (IBW: 2.52 ± 0.11 g).

Diet	CTRL	DON 5	DON 11	P value
Survival, %	100.0 ± 0.0	99.0 ± 1.2	97.0 ± 3.5	0.103
FBW, g	9.39 ± 0.24 ^{c}	5.83 ± 0.25 ^{b}	3.90 ± 0.23 ^{a}	< 0.001
SGR, % d^{-1}	4.53 ± 0.09 ^{c}	2.90 ± 0.16 ^{b}	1.50 ± 0.23 ^{a}	< 0.001
FCR	0.96 ± 0.02 ^{a}	1.08 ± 0.03 ^{a}	1.56 ± 0.19 ^{b}	< 0.001
FI, %ABW d^{-1}	3.81 ± 0.12 ^{c}	2.93 ± 0.14 ^{b}	2.13 ± 0.15 ^{a}	< 0.001
PER	2.18 ± 0.04 ^{c}	1.93 ± 0.06 ^{b}	1.35 ± 0.17 ^{a}	< 0.001

Values are means and standard deviation (n = 4).
Different superscripts within a row denote a statistical difference ($P < 0.05$).

Table 3
Growth performance after 60 days of feeding (IBW: 2.52 ± 0.11 g).

Diet	CTRL	DON 5	DON 11	P value
Survival, %	99.5 ± 1.0 ^{b}	98.0 ± 2.3 ^{b}	94.0 ± 2.8 ^{a}	0.020
FBW, g	24.77 ± 0.86 ^{c}	12.52 ± 0.83 ^{b}	5.96 ± 0.54 ^{a}	< 0.001
SGR, % d^{-1}	3.81 ± 0.06 ^{c}	2.67 ± 0.12 ^{b}	1.43 ± 0.15 ^{a}	< 0.001
FCR	1.07 ± 0.03 ^{a}	1.12 ± 0.03 ^{a}	1.58 ± 0.23 ^{b}	0.001
FI, %ABW d^{-1}	2.90 ± 0.10 ^{c}	2.47 ± 0.11 ^{b}	1.99 ± 0.16 ^{a}	< 0.001
PER	1.96 ± 0.06 ^{b}	1.88 ± 0.06 ^{b}	1.35 ± 0.18 ^{a}	< 0.001
HSI, %	1.62 ± 0.07 ^{a}	2.47 ± 0.07 ^{b}	2.19 ± 0.30 ^{b}	< 0.001

Values are means and standard deviation (n = 4).
Different superscripts within a row denote a statistical difference (P < 0.05).

11) showed a significantly lower FBW, SGR and FI than those fed with the DON 5 diet (P < 0.001). Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet (P = 0.001). The HSI varied between 1.62 and 2.47, with CTRL fish showing a significantly lower HSI than those fed both DON supplemented diets (DON 5 and DON 11; P < 0.001; Table 3).

3.2. Whole-body composition

The whole-body composition of fish in terms of moisture, fat and energy was not affected by the various dietary treatments (P > 0.05, Table 4). However, fish fed the CTRL diet showed a significantly lower whole-body ash and a significantly higher whole-body protein than those fed both DON supplemented diets (DON 5 and DON 11; P < 0.001).

3.3. Whole-body nutrient retention and apparent digestibility

Protein retention varied between 17.83 and 32.56%, and fish fed the CTRL diet showed a significantly higher protein retention than those fed both DON supplemented diets (DON 5 and DON 11) (P < 0.001; Table 5). Moreover, fish fed the highest DON dose (DON 11) showed a significantly lower protein retention than those fed with the DON 5 diet (P = 0.001). Fat retention ranged from 58.98 to 81.86%, while energy retention varied between 31.09 and 40.16%. Fish fed the CTRL and DON 5 diets showed a significantly higher fat and energy retention than those fed the DON 11 diet (P < 0.001; Table 5). Digestibility of dry matter, protein and energy was not affected by dietary treatments (P > 0.05; Table 6).

Regarding, DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) analysed in trout's faeces, only DON and DON-3-sulfate were detected (Fig. 1). No DON or DON metabolites were detected in animals fed the control diet (CRTL). In the faeces from treatment DON 5 and DON 11, 13.2% and 10.5% of DON was recovered, respectively, compared to the total ingested DON. The high solubility of DON in water may explain such low recovery rates. More than 80% of the recovered mycotoxin in faeces, was in the form of DON-3-sulfate (DON 5 = 84.07% and DON 11 = 82.09% of DON-3-sulfate) and around of 15% of DON (DON 5 = 15.93% and DON 11 = 17.91% of DON).

3.4. Enzyme activity analyses

The results of total pepsin, trypsin, chymotrypsin, amylase and lipase activities measured in the different experimental groups are shown in Fig. 2 (i to v, respectively). For total pepsin, DON 11 showed a higher activity (0.0021 ± 0.0003 U g^{-1} BW; P < 0.001) when compared to the CTRL (0.0012 ± 0.0002 U g^{-1} BW) and DON 5 (0.0013 ± 0.0003 U g^{-1} BW) groups. In contrast, trypsin, showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g^{-1} BW; P = 0.043) being statistically lower than DON 11 (2.40 ± 1.19 U g^{-1} BW), however, statistically similar (P > 0.05) to the control (2.58 ± 2.08 U g^{-1} BW). Chymotrypsin did not present significant differences (P > 0.05) among the different experimental groups. DON 11 presented a higher lipase activity (0.0022 ± 0.0001 U g^{-1} BW; P = 0.002) compared to CTRL (0.0005 ± 0.00001 U g^{-1} BW) and DON 5 (0.0013 ± 0.0007 U g^{-1} BW). Amylase did not present significant differences (P > 0.05) among the different dietary groups.

3.5. Gene expression

The relative expression levels of gene transcripts for stress regulation (*star*; head kidney; *crf1*, *crf2*, *crfbp*; brain), growth control (*igf1*, *igf2*; liver; *adcyap1a*; brain), enzymatic digestion, regulation and appetite control (*sst2*, *chia*, *pga*, *lpl*, *ghrl*, *cel1*, *cel2*, *cckt*, *cckn*, *cckl*, *amy2a1*, *atp4a*, *crtl*, *try1*, *try2*, *try3*; GIT; *lep*, *npy*; brain) are shown in Table 7. Expression of *igf1* and *igf2* was significantly lower in DON 5 and DON 11 when compared to CTRL group (P = 0.004 and P = 0.008, respectively). Interestingly, expression levels of *npy* and *adcyap1a/PACAP* mRNAs were significantly up-regulated by DON treatments (P = 0.004 and P = 0.005, respectively). In contrast *star* mRNA transcripts displayed a trend toward higher abundance in both DON fed treatments, however this was not significant (P = 0.088). Analyses of specific mRNA transcript levels across all enzyme precursors (*crtl*, *Try1*, *Try2*, *Try3*), highlighted *try3* to be significantly up-regulated (P = 0.036) in both DON fed groups.

4. Discussion

The impact of DON on fish has been further elucidated in recent years with the rainbow trout as a useful model. However, very little is known in comparison to land-farmed animals. This is especially true regarding diagnostic parameters to correctly identify the impact of DON ingestion in a production setting. For this purpose, an experimental protocol was designed to further understand the impact of DON on growth performance and also to explore the underpinning causes for the reported decreases in growth performance. The DON effect was evaluated by studying the regulation of digestion, both at the enzyme activity level and through mRNA gene expression, where we surveyed stress regulation, growth and appetite control processes. In order to investigate metabolic breakdown of DON, faeces was analysed for DON metabolites.

Growth performance was affected by DON (4714 ± 566 and 11,412 ± 1141 μg kg^{-1}), in a similar manner to that previously described (Hooft et al., 2011; Matejova et al., 2015; Ryerse et al., 2015), despite higher DON concentrations. Fish fed DON showed a significantly lower FBW, SGR and FI when compared to the control (P < 0.001). Moreover, the highest DON dose (DON 11) showed a significantly lower FBW, SGR and FI than the dose used in the DON 5 diet (P < 0.001). In addition, FCR was significantly increased in fish fed the DON 11 diet (P = 0.001). The HSI was significantly higher in animals fed DON, although this difference was not observed during dissection or visual examination for clinical signs. Interestingly, no macroscopic lesions were found (e.g. internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to

Table 4
Whole-body composition.

Diet	CTRL	DON 5	DON 11	P value
Moisture, %	63.53 ± 0.95	63.73 ± 0.78	64.36 ± 0.64	0.353
Protein, %	16.67 ± 0.52 ^{b}	15.22 ± 0.45 ^{a}	15.09 ± 0.24 ^{a}	0.001
Fat, %	14.06 ± 0.65	13.95 ± 0.28	13.65 ± 0.52	0.534
Ash, %	5.05 ± 0.26 ^{a}	6.03 ± 0.18 ^{b}	6.13 ± 0.09 ^{b}	< 0.001
Energy, kJ g^{-1}	8.50 ± 0.26	8.51 ± 0.15	8.46 ± 0.29	0.953

Initial fish values: moisture 65.30%; protein 17.14%; fat, 11.97%; ash 5.11%; energy 6.63 kJ g^{-1}.
Values are means and standard deviation (n = 4), after 60 days of feeding.
Different superscripts within a row, denotes a statistical difference (P < 0.05).

Table 5
Whole-body nutrient retention.

Diet	CTRL	DON 5	DON 11	P value
Protein, %	32.56 ± 1.21 ^{c}	27.40 ± 1.03 ^{b}	17.83 ± 2.40 ^{a}	< 0.001
Fat, %	81.86 ± 4.32 ^{b}	78.98 ± 0.64 ^{b}	58.98 ± 10.59 ^{a}	0.001
Energy, %	40.16 ± 0.68 ^{b}	39.41 ± 1.15 ^{b}	31.09 ± 3.59 ^{a}	0.001

Values are means and standard deviation (n = 3), after 60 days of feeding.
Different superscripts within a row denote a statistical difference (P < 0.05).

Table 6
Apparent digestibility of dry matter, protein and energy.

	CTRL	DON 5	DON 11	P-value
Dry matter, %	70.0 ± 3.1	68.7 ± 1.7	68.4 ± 1.8	0.576
Protein, %	89.1 ± 1.2	88.5 ± 0.7	88.4 ± 0.7	0.499
Energy, %	82.2 ± 2.0	81.5 ± 1.1	81.3 ± 0.4	0.621

Values are means and standard deviation (n = 4), after 60 days of feeding.

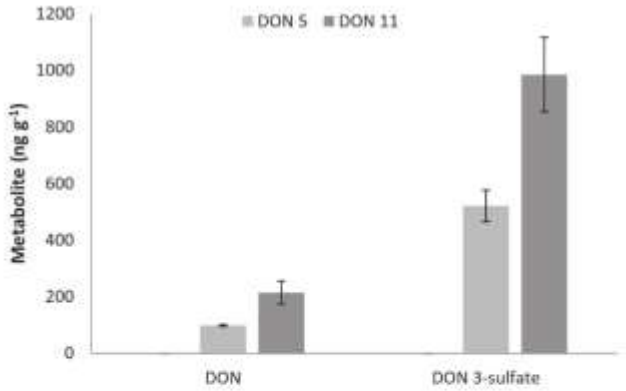

Fig. 1. Quantity of DON and DON 3-sulfate in ng per g of faeces for treatment DON 5 and DON 11. No DON or DON-metabolites were found in the control treatment, after 60 days of feeding. DOM and DOM 3-sulfate were not identified in the samples collected. Values are means and standard deviation of the mean (n = 8).

fins) were detected on animals fed DON, confirming that diagnosis of DON ingestion is extremely difficult, even at high dosages (DON 11; 11,412 ± 1141 μg kg^{-1}). According to previous studies, the impact of DON might vary greatly depending on unknown factors, even for the same species. For the same range of DON contamination (0.3 to 5.9 ppm), some authors (Hooft et al., 2011; Ryerse et al., 2015) did not find any major pathological changes in the distal intestine of trout, while in other situations, gastrointestinal haemorrhages were found (Matejova et al., 2014). In addition to the lower growth in the DON fed treatments, which was visually detected (Fig. S1), DON 11 treated fish also had significantly lower survival rates. In our opinion, it is highly unlikely under production conditions that such an increase in mortality/decrease in performance would be associated to DON ingestion particularly when specific subclinical signs are lacking. The present scenario illustrating the significant impact of DON in trout coupled to a complete lack of clinical symptoms highlights the need for further investigation to support an early diagnosis for DON ingestion.

Anti-nutritional factors that decrease enzymatic activity, or form complexes with proteins thereby modifying digestion processes have been described (Santigosa et al., 2008) (Moyano et al., 1999). However, very little is known about the impact of mycotoxins on digestive enzymes and information regarding the impact of DON is very scarce. For AF ingestion, Han et al. (2008) observed increased protease, amylase, chymotrypsin, and trypsin activity and an apparent decrease in digestibility of crude protein in 42-day old ducks fed 0.02 and 0.04 mg kg^{-1} AF. To our knowledge most studies have been focused on the effects of DON on the nutrient absorption process (Grenier and Applegate, 2013), however there is no information available regarding the effects of DON on digestive enzymes.

The major contributing factor to the conversion of feed to growth is protein turnover thus proteases play an essential role. Proteolytic enzyme activity (pepsin, trypsin and chymotrypsin) was significantly altered in DON fed groups. Total pepsin activity was significantly higher in the DON 11 group (0.0021 ± 0.0003 U g^{-1} BW; P < 0.001) compared to the CTRL (0.0012 ± 0.0002 U g^{-1} BW) and DON 5 (0.0013 ± 0.0003 U g^{-1} BW) groups. However, the observed impact upon pepsin might be directly related to the decrease of feed intake in this group, and not necessarily a direct impact of DON on pepsin or any pepsin precursor. It is well described that pepsinogen is rapidly synthesised during feeding and then secreted, whereupon pepsin activity increases. For example, Einarsson et al. (1996) observed that under starvation conditions in *Salmo salar* there was a slight rise in pepsin activity in the stomach mucosa suggesting that pepsin can be stored in salmonids. Therefore, and taking into account that animals were fed 3 h prior to sampling, we cannot exclude the hypothesis that higher pepsin secretion in the DON 11 group could be related to lower feed intake. This in turn may result in a retention of pepsin in stomach mucus, due to a markedly reduced stomach evacuation. Chymotrypsin did not show significant differences between treatments, although a numerically lower activity was observed in the DON 11 group. Rungruangsak-Torrissen et al. (2006) observed a higher specific activity of chymotrypsin when growth was limited or depressed due to starvation or food deprivation. However, considering that feed intake was reduced in the DON 11 group, possibly due to hormonal regulation, no major effect was observed in chymotrypsin activity. As trypsin activates chymotrypsin in fish (Sunde et al., 2001) it is difficult to find out if DON impacted, directly or indirectly, this activation. Trypsin showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g^{-1} BW; P = 0.043), statistically insignificant when compared to DON 11 (2.40 ± 1.19 U g^{-1} BW) but similar to CTRL (2.58 ± 2.08 U g^{-1} BW). Also interesting is the observation that from the four types of alkaline proteases (*ctrl*; *try1*, *try2* and *try3*), only mesotrypsinogen (trypsinogen-3) showed a higher mRNA expression level in DON fed treatments. Mesotrypsin is a specialized protease known for its resistance to trypsin inhibitors. It is thought to play a special role in the degradation of trypsin inhibitors, possibly to help with the digestion of inhibitor-rich plant meals such as soybeans and lima beans, which might be the case of plant meals containing DON as well (Szmola et al., 2003), however more research is needed on this topic.

Both trypsin activity and mRNA expression levels for *igf1* and *igf2* were found to be significantly higher in the CTRL when compared to DON 5 and DON 11. It is well known that trypsin cleaves protein at the carboxyl side of the basic amino acids lysine and arginine (Stryer, 1988), which elevates plasma insulin levels in salmonids (Plisetskaya et al., 1991). In turn, insulin stimulates amino acid uptake and protein synthesis especially in the muscle tissue (Matty, 1986; Murat et al., 1981), leading to a growth promoting effect in salmonids (Donaldson et al., 1979). Proteolytic enzyme differences observed between the treatments were probably not associated with secretion control, as cholecystokinin-like peptides (*cck-t; cck-n or cck-l*) did not significantly alter due to DON treatment.

Apparent digestibility of dry matter, protein and energy in the present study was not affected by dietary levels of DON, which agrees with a previous study in trout (Hooft et al., 2011). In current literature, contradictory information about the effects of DON on ADC has been reported. While, for example, Dänicke et al. (2004) and Van Le Thanh et al. (2015) reported that DON could affect crude protein digestibility in piglets, Jo et al. (2016) reported no differences in ADC in growing pigs fed 10,000 μg kg^{-1} DON. The latter is in agreement with findings from the present study for similar levels of contamination. Jo et al. (2016) reported that DON contamination might affect essential amino

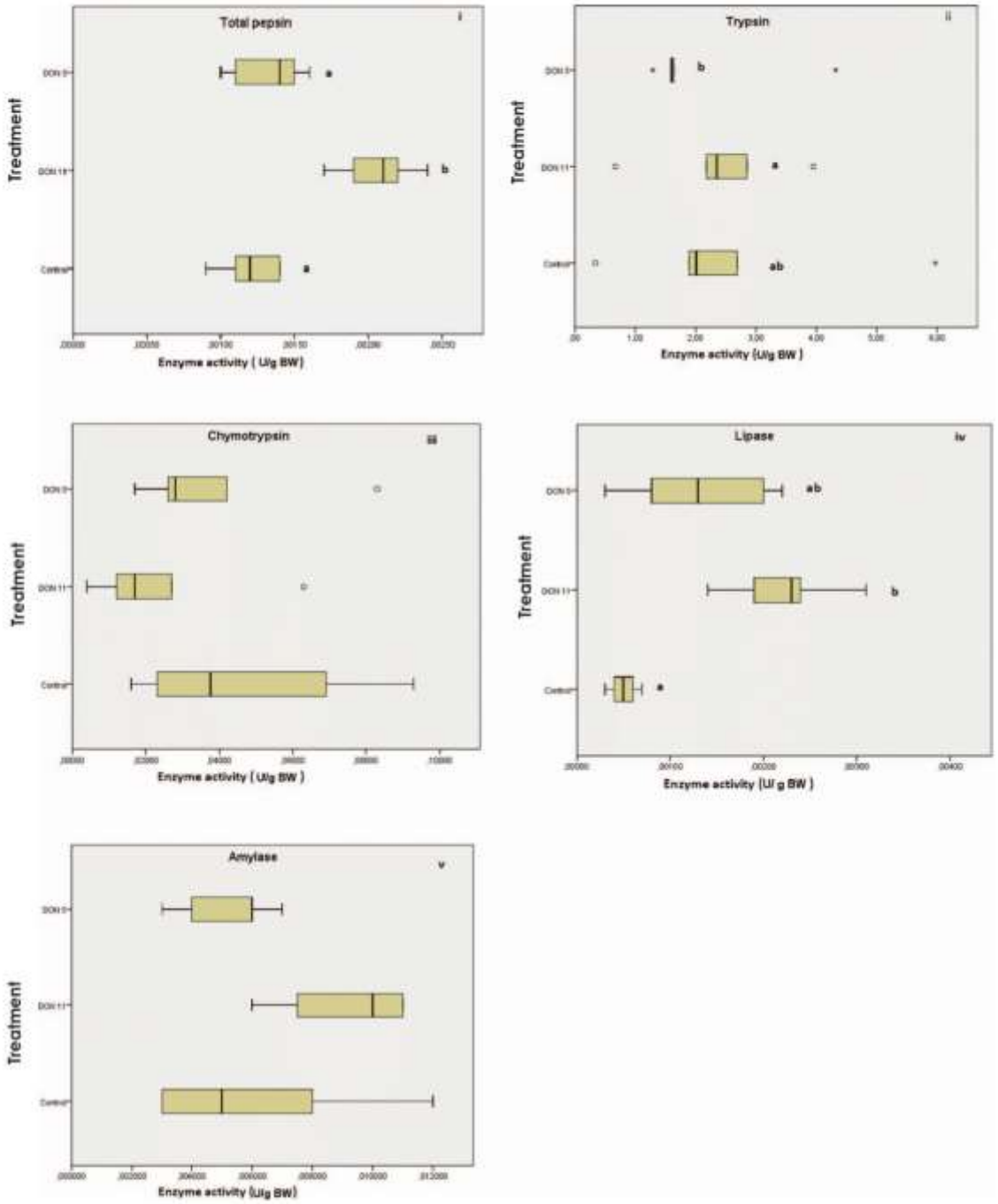

Fig. 2. Enzyme activity of the different experimental groups after 60 days of feeding, expressed in U g^{-1} body weight. Results of total pepsin (i), trypsin (ii), chymotrypsin (iii), amylase (iv) and lipase (v). Different superscripts letters, denotes a statistical difference ($P < 0.05$). Values ($n = 5$) are displayed as the lower (Quartile1) and upper (Quartile 3) quartiles, the median and data outliers (•).

acid digestibility. In the present study, it was observed that DON affected trypsin, and consequently trypsin may influence the levels of insulin, which will ultimately influence amino acid uptake. However, this requires further research to verify whether DON directly affects this pathway.

In this study protein, fat and energy retention were all significantly affected in animals fed DON. The low performance of the animals fed DON could be a consequence of decreased nutrient uptake and transport rather than lower nutrient digestibility, as enzyme activity and ADC appear unaffected by DON. The reason behind the enzyme activity differences among the experimental groups is not clear, however differential feed intake may influence our interpretation.

The neuroendocrine process that controls satiety is regulated, with others, by Neuropeptide Y, Leptin, Ghrelin or Adenylate cyclase-activating polypeptide (PACAP). In the present work, leptin and ghrelin mRNA transcripts were not influenced by DON which was not surprising as Leptin activity is related to long-term regulation of energy balance, suppressing food intake, while Ghrelin is a fast-acting hormone

Table 7
Relative expression levels of genes tested for the three experimental treatments.

Tissue	Genes	CTRL	DON 5	DON 11	P-value
Head kidney	*star*	0.36 ± 0.14	2.87 ± 1.10	2.26 ± 0.77	0.088
Liver	*lgf1*	0.95 ± 0.16 [a]	0.43 ± 0.08 [b]	0.49 ± 0.04 [b]	0.004
	lgf2	0.90 ± 0.23 [a]	0.41 ± 0.06 [b]	0.25 ± 0.06 [b]	0.008
Brain	*crf1*	1.03 ± 0.07	1.19 ± 0.26	1.22 ± 0.16	0.726
	crf2	1.02 ± 0.07	1.12 ± 0.10	1.05 ± 0.08	0.660
	crfbp	0.94 ± 0.09	0.79 ± 0.15	0.93 ± 0.07	0.555
	npy	0.94 ± 0.14 [a]	1.59 ± 0.18 [b]	1.67 ± 0.30 [b]	0.004
	adcyap1a	0.99 ± 0.08 [a]	1.40 ± 0.11 [b]	1.44 ± 0.11 [b]	0.005
	lep	0.94 ± 0.09	0.95 ± 0.17	1.11 ± 0.18	0.640
GIT	*sst2*	0.90 ± 0.08	0.70 ± 0.18	0.82 ± 0.10	0.489
	chia	0.96 ± 0.11	1.10 ± 0.20	1.28 ± 0.25	0.497
	pgo	0.97 ± 0.17	0.88 ± 0.17	0.78 ± 0.10	0.640
	lpl	1.17 ± 0.22	1.74 ± 0.38	1.03 ± 0.11	0.122
	ghrl	0.88 ± 0.19	1.54 ± 0.34	1.20 ± 0.16	0.127
	cel1	1.25 ± 0.26	1.29 ± 0.29	0.83 ± 0.14	0.293
	cel2	0.94 ± 0.16	0.80 ± 0.24	0.59 ± 0.09	0.313
	cckr	0.84 ± 0.16	0.89 ± 0.16	0.70 ± 0.14	0.612
	cckn	1.25 ± 0.28	1.35 ± 0.15	1.83 ± 0.39	0.291
	cckl	0.99 ± 0.19	1.14 ± 0.08	1.18 ± 0.21	0.706
	amy2a	1.05 ± 0.15	1.10 ± 0.27	1.00 ± 0.16	0.929
	atp4a	1.00 ± 0.14	1.41 ± 0.27	1.09 ± 0.16	0.278
	crtl	1.06 ± 0.21	1.01 ± 0.19	0.68 ± 0.10	0.228
	try1	0.88 ± 0.23	0.70 ± 0.23	0.64 ± 0.12	0.653
	try2	1.22 ± 0.29	1.25 ± 0.24	0.95 ± 0.15	0.612
	try3	1.00 ± 0.15 [a]	2.03 ± 0.43 [b]	1.90 ± 0.25 [b]	0.036

Values are means and standard deviation of the mean (n = 7) after 60 days of feeding.
Different superscripts within a row denote a statistical difference (P < 0.05).

acting as "stopper" after meal initiation (Klok et al., 2007). In contrast, PACAP plays an important and direct role in the regulation of feed intake. In goldfish, it has been observed that intracerebroventricular injections of PACAP suppress food intake (Matsuda et al., 2005). In the present study, upregulation of *adcyap1a* or PACAP mRNAs provides a possible link to the observed reduction in feed intake, as described in the literature (Chance et al., 1995; Li et al., 2015; Morley et al., 1992; Mounien et al., 2008; Tachibana et al., 2003). In zebrafish, PACAP greatly decreases the frequency of gut motility waves (Holmberg et al., 2004) which might also have an impact on nutrient absorption. *npy* was also upregulated in DON fed treatments, however its putative role in our experimental setup is challenging to explain. In mammals, *npy* is a key factor in the regulation of feeding behaviour and there is strong evidence of a direct physiological role of *npy* and its expression levels in controlling feed intake (Chamorro et al., 2002). However, most of the studies published suggest that elevated brain *npy* levels induce increased feed intake (see review Chamorro et al., 2002), which is contrary to the obtained results in this study. Though, in the present study the upregulation of *npy*, seems to be a consequence of the reduce feed intake, i.e., *npy*, as explained by Narnaware and Peter (2001), is regulated in part by the feeding state of the animal, since food deprivation induces a marked increase in both *npy* and its mRNA levels in the brain. Narnaware and Peter (2001) observed an increased *npy* mRNA expression in several brain regions of goldfish in response to food deprivation, which might help to explain the findings of the present study.

Metabolism of DON could also explain the lack of any lesions in trout when compared, for example, with swine and poultry. Metabolism of trichothecenes in several livestock species has been reported however, these studies focus on the formation of de-epoxy-DON or on glucuronidation (Dänicke et al., 2004; Eriksen et al., 2002; Schwartz-Zimmermann et al., 2015). While de-epoxy-DON is achieved mainly by gut microbiota, glucuronidation is carried out by endogenous UDP-glucuronosyltransferases in the liver, and possibly also in intestinal microsomes (Maul et al., 2015). Metabolism pathways of DON vary greatly within species (Schwartz-Zimmermann et al., 2015). In fish, only one report in brown bullhead catfish (*Ameiurus nebulosus*) has shown the capability of the gut microbiota of this species to biotransform trichothecenes into their de-epoxy forms (Guan et al., 2009). DON can also be metabolized by sulfation, which was only recently discovered as a major pathway for chickens and turkeys (Devreese et al., 2015; Schwartz-Zimmermann et al., 2015; Wan et al., 2014). In the present study, it has been shown for the first time in rainbow trout that DON is metabolized into DON-3-sulfate, which is less toxic than DON (Schwartz-Zimmermann et al., 2015). Despite considerable mycotoxin leaching from the faeces, due to the high solubility of DON/DON-metabolites in water, > 80% of the mycotoxin in faeces was recovered in the form of DON-3-sulfate. The location of formation, absorption and elimination of DON-3-sulfate is not known and was not further investigated in the present trial. However, as suggested by (Schwartz-Zimmermann et al., 2015), DON might be metabolized to DON-3-sulfate in the intestinal mucosa, liver, or even in the kidney as happens for some other vertebrate species. The formation of DON-3-sulfate, could also explain the absence of major clinical signs in trout fed DON, particularly at DON 5 treatment levels, which still had a considerable impact on feed intake but did not cause major lesions (external macroscopic observations).

In the present work, the impact of DON on the GIT or on the absorptive process was not evaluated. The potential impact of mycotoxins on the GIT in livestock species is well described (see reviews; Broom, 2015; Grenier and Applegate, 2013). Due to the mode of action of DON (as an inhibitor of protein synthesis) and the high rate of protein turnover in intestinal cells, it is to some extent also expected to observe altered intestinal areas in trout. However, the literature is not consistent when reporting the impact of DON on the trout GIT (Hooft et al., 2011; Matejova et al., 2014; Ryerse et al., 2015). Despite only being evaluated macroscopically, any potential microscopic intestinal damage caused by DON and the consequent influence on nutrient absorption cannot be discarded. However, the novel fact that 80% of the recovered DON is metabolized into DON-3-sulfate might help to explain the lack of consistency in GIT damage in trout. The elucidation of metabolism pathways in fish in respect to DON would be a major step toward understanding the underpinning mechanisms of sensitivity/resistance to this mycotoxin in fish.

5. Conclusion

Deoxynivalenol exposure in fish has been characterised mainly by reduced feed intake and growth performance. Contrary to land farm animals, DON ingestion in fish does not lead to specific clinical signs, except anorexia (at high dosages, above 5 ppm), and some minor altered blood parameters (blood parameters (erythrocyte/leucocyte count), blood enzymes (ALT, AST or ALP), liver alterations or immune parameters suppression) which are generally not specific for DON-induced mycotoxicosis. In the present study, it was observed that digestive enzymes (regarding activity and mRNA expression) are affected; however, we were unable to clarify if this was caused by DON ingestion or by suppression of feed intake. A pair-fed treatment would be useful in the future in order to distinguish between effects from DON intake and any possible effects from suppression of feed intake. Moreover, nutrient (protein, fat and energy) retention was affected by dietary DON suggesting that nutrient uptake and transport might be affected. In the present study the upregulation of npy, seems to be a response to food deprivation, but in contrast upregulation of PACAP seems to be fundamental for explaining the reduction of feed intake in DON fed treatments, inducing anorexia. Further research is needed focusing on the effect of DON on appetite control by addressing the influence of DON on the hypothalamic melanocortin system. Suppression of appetite due to DON contamination in feeds might be a defense mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The discovery of DON-3-sulfate as a novel trout metabolite makes it a potential biomarker of DON exposure. However, further characterisation of its toxicological relevance is essential.

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Contribution

The experimental design was from the responsibility of RAG, DS, GMR and SM. In vivo experiment and digestibility measurement was at the responsibility of JD and PR. Enzyme activities were performed by CN, MY and FJM. Gene expression were performed by RAG, NG, GMR. Mycotoxin metabolites were analysed by GB and TC. Results analyse, writing, statistical treatment and interpretation of the data, was the responsibility of RAG with the active collaboration of all co-authors. All authors have approved the final article and contributed for its revision.

Declaration of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.03.001.

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- **CHAPTER 4 - Fate of [^{3}H]-deoxynivalenol in rainbow trout (*Oncorhynchus mykiss*) juveniles: tissue distribution and excretion was based on:**

Fate of [^{3}H]-deoxynivalenol in rainbow trout (Oncorhynchus mykiss) juveniles: tissue distribution and excretion

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Fate of [^{3}H]-Deoxynivalenol in Rainbow Trout (*Oncorhynchus mykiss*) Juveniles: Tissue Distribution and Excretion

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Abstract

Deoxynivalenol (DON), a *Fusarium* mycotoxin, is one of the most prevalent mycotoxins in aquafeeds. The toxicokinetics of DON are rarely studied in aquatic species. The present study used juvenile rainbow trout (*Oncorhynchus mykiss*) with a mean initial body weight of 7.72 ± 1.42 g in order to evaluate the pharmacokinetic behaviour and the metabolization of radiolabelled DON ([^{3}H]-DON). In a first trial, 30 fish were tube-fed with four pellets containing a total of 125 ± 0.019 ng of [^{3}H]-DON. At different sampling time points after feeding (1 h, 3 h, 6 h, 12 h or 24 h), the tissue distribution of the [^{3}H]-DON was assessed by liquid scintillation counting. In a second trial, five fish were tube-fed four pellets containing a total of 663 ng of unlabelled-DON. Twenty-four hours after feeding, metabolites of DON excreted into the water were analysed by LC-MS/MS. [^{3}H]-DON was detected in fish liver one hour after tube-feeding, indicating a rapid absorption of DON. In the first hour, [^{3}H]-DON achieved its maximum in the gastro-intestinal tract (GIT) (20.56 ± 8.30 ng). However, 6.19 ± 0.83 ng of [^{3}H]-DON was also detected in the water at this sampling time point. The fast excretion of [^{3}H]-DON (above the average gastric emptying time of trout) might be related to its high-water solubility and consequent excretion with the fluid phase of the chyme. The amount of [^{3}H]-DON in the GIT was stable during the first six hours. Such long transit time of DON through the GIT increases the potential for damage and absorption. The period between six and twelve hours seems to be the turning point in terms of DON excretion. Twelve hours after tube-feeding, the trout excreted 50.71 ± 22.17% of the tube-fed DON amount into water, while at the previous sampling time point (six hours) only 11.03 ± 6.09% were detected. These data suggest that an effective method for gastrointestinal DON detoxification in trout requires a period of action lower than six hours. In the present trial, no DON metabolites were detected in water.

Keywords: *Fusarium* mycotoxin; Toxicokinetics; Deoxynivalenol (DON); Tube-feeding

Introduction

Fusarium mycotoxins are the most prevalent mycotoxins found in aquafeeds, reflecting the type and inclusion levels of plant meals used in these diets [1-3]. Moreover, the presence of secondary metabolites of *Fusarium* spp. are expected to increase in aquaculture raw materials in response to climate change [4-6], which might represent a challenge for the aquaculture industry. Among the metabolites produced by the genus *Fusarium*, deoxynivalenol (DON) is reported to be the main mycotoxin found in small grain cereals [7,8]. Absorption, distribution, metabolism and elimination (ADME) of DON differs among animal species [9]. The toxic effects and toxicokinetics of DON are well described for land farmed animals [9], but less is known for aquatic animals. Only recently, Bernhoft et al. [10] evaluated the tissue distribution and elimination of DON in Atlantic salmon (*Salmo salar*), considering also the possibility of accumulation of mycotoxins or their metabolites in fish tissues. Bernhoft et al. [10] reported that DON was present in liver, kidney, muscle, skin and brain of Atlantic salmon after treatment with 6 mg kg^{-1} DON for eight weeks. For aquatic animals almost, nothing is known about metabolisation of DON. However, for terrestrial animals it was observed that DON can induce phase I and II liver biotransformation enzymes [11]. Advancements in knowledge of DON toxicokinetics and metabolisation in fish will support risk assessment of DON for aquatic species and its counteraction. Taking into account that little is known about the fate of DON in fish, especially with regard to excretion and biotransformation, the objective of the present study was to evaluate the pharmacokinetic behaviour of radio-labelled DON ([^{3}H]-DON) in rainbow trout (*Oncorhynchus mykiss*), focusing on tissue distribution, excretion and possible DON biotransformation.

Materials and Methods

Ethics statement on animal experiments

All experimental procedures involving animals followed the EU Directive 2010/63/EU and National Decreto-Lei 113/2013 legislation for animal experimentation and welfare. Animal handling and experiments were performed by qualified operators accredited by the Portuguese *Direção-Geral de Alimentação e Veterinária* (DGAV). This study was conducted at the Center for Marine Sciences (CCMAR) of Universidade do Algarve, Faro, Portugal.

Husbandry and fish nutritional background

Juvenile rainbow trout (*Oncorhynchus mykiss*) with a mean initial body weight of 7.72 ± 1.42 g were acclimatised in 40 L cylinder-conical fiberglass tanks over the course of three weeks. During the acclimatisation period, fish were fed a mycotoxin-free diet at an amount corresponding to 1.5% body weight, four times a day via automatic feeders. Fish were kept at a density of less than 2 kg m^{-3}, in

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a recirculation freshwater system at 15 ± 1.0°C, with a 12 h Light: 12 h Dark photoperiod. Dissolved oxygen levels were kept above 90% oxygen saturation.

Pellets labelled with [^{3}H]-deoxynivalenol

For the metabolic trial, each feed pellet was individually labelled with 31.25 ng of the tracer, [^{3}H]-DON (3.7 MBq; American Radiolabeled Chemicals Inc., The Netherlands). After labelling, the pellets were dried at 50°C for 30 minutes and stored at 8°C for the subsequent tube-feeding procedure.

Experimental procedure

After the acclimatisation period, rainbow trout juveniles were tube-fed with pellets containing radiolabelled DON. Tube-feeding was performed according to the method described by Rust et al. [12], modified by Costas [13]. Randomly selected fish (n=6, for each sampling time point) were transferred to the laboratory after being fasted for 18 h. In brief, fish were anesthetised (ethyl 3-aminobenzoate - MS-222, Sigma) and tube-fed with four pellets of the diet each (corresponding to 0.13% body weight and a total of 125 ± 0.019 ng of [^{3}H]-DON). For tube-feeding, a hollow plastic tube of 1.5 mm inner diameter and a solid piece with a smaller diameter placed inside as a plunger were used. The diameter and length of the plastic tubing was previously tested to avoid injuring the oesophagus of the rainbow trout juveniles. Tube-fed fish were allowed to recover for 10 minutes in clean, fresh water to eliminate any residual anaesthetic from the skin and gills and monitored for possible pellet regurgitation. After this period, fish were transferred to the incubation chamber (individually housed) tempered to 15 °C. Each chamber (2 litres) was hermetically sealed and supplied with a gentle oxygen flow. After the incubation period (1 h, 3 h, 6 h, 12 h or 24 h; 6 incubation chambers), oxygen flow was stopped, and fish were sacrificed inside the chambers using a lethal dose of the anaesthetic (ethyl 3-aminobenzoate - MS-222, Sigma).

After fish removal, water from each incubation chamber was collected (5 mL aliquots per chamber) for radioactive counting to infer the quantity of mycotoxin excreted by the fish. Fish were individually weighed and sampled for muscle (without skin), skin, liver, kidney and gastro-intestinal tract (GIT). All samples were weighed, except those from the kidneys due to its low weights and the absence of accurate scales in the radioactivity laboratory. Muscle and skin samples were not collected from fish incubated for one hour, as digestion is assumed to take longer than 1 hour, and therefore, radioactivity was not being expected to reach these tissues. Blood was not collected due to the small size of the fish and coagulation of the blood after the anaesthetic overdose.

Mycotoxin fate determination

Samples of the dissected tissues were completely dissolved in Solvable™ (Perkin Elmer, USA) at 50°C for 24 h. Radioactivity in tissue samples (muscle, skin, liver, GIT), water samples and pellets (n=50; to confirm labelling success) were quantified by scintillation counting in a Tri-Carb 2910TR low activity liquid scintillation analyser (Perkin Elmer, USA) after addition of Ultima Gold XR scintillation cocktail (Perkin Elmer, USA). The metabolic budgets were calculated after subtraction of blanks for quench and lumex correction.

Deoxynivalenol metabolisation assay

For the DON metabolisation study, each pellet was supplemented with 165.75 ng of unlabelled DON, giving a total dose of 663 ng of DON per fish (four pellets per tube-fed animal). After adding DON, the pellets were dried at 50°C for 30 minutes and then left to cool at room temperature before the subsequent tube-feeding procedure. The experimental procedure was performed as for the [^{3}H]-DON treatment, with the only difference being that water was the only sample collected from the metabolic chambers, after 24 h of incubation, in order to identify and quantify excreted DON and its potential metabolites. No fish tissues were analysed during this assay. Water collected from the chamber (50 mL aliquots from each chamber), was analysed by LC-MS/MS for the presence of DON and potential DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) as described by Streit et al. [14].

Determination of toxicokinetic parameters

Toxicokinetic parameters were determined for GIT and liver collected during the [^{3}H]-DON experiment. All calculations were based on the assumption that 1 DPM equals 0.22 pg of mycotoxin. DON concentration at zero time (C_0) was determined from the tissue concentration–time curves obtained. Elimination constants (K_{el}) were determined by curve regression ($C(t)=c_0*e^{(K_{el}*t)}$). The elimination half-life, the time necessary to half the concentration, was calculated as in $t_{1/2}=\ln 2/K_{el}$. C_{max} is the peak of [^{3}H]-DON concentration in the respective tissue at a certain time (t_{max}). Toxicokinetic parameters were not determined for skin and muscle due to the insufficient sampling points.

Statistical Analysis

Results are presented as means ± standard deviation (S.D.). Results expressed as percentage were arcsine-transformed prior to statistical analysis. Statistical analyses were performed using the STATISTICA version 8.0 software (StatSoft Inc.). Data were verified for normal distribution and homogeneity of variances. Significant differences between groups (samples taken at the same time point) were assessed by one-way ANOVA. When significant differences were detected, the Tukey's multiple-comparison test was used to assess differences between groups. Differences were considered to be significant when $p<0.05$.

Results

Deoxynivalenol distribution and excretion

Pellets presented a mean value of 151,282 disintegrations per minute (DPM). Results for mycotoxin fate in rainbow trout are expressed based on the assumption that 1 DPM equals 0.22 pg of DON. The DON distribution in fish tissue (ng of DON; GIT, liver, kidney, muscle, kidney and skin) and in water, for each sampling point, and the percentage of DON in tissues (sum of tissues per sampling point) or in water relatively to tube-fed amount (125 ± 0.019 ng DON) is shown in Table 1. One hour after tube-feeding, [^{3}H]-DON was detected mainly in the GIT (20.56 ± 8.30 ng), and low levels were detected in the liver (1.44 ± 0.67 ng) and kidneys (0.23 ± 0.13 ng) (muscle and skin were not sampled at one-hour post tube-feeding). At one-hour sampling time point, 6.19 ± 0.83 ng [^{3}H]-DON was detected in the water (Table 1). At this sampling point [^{3}H]-DON in water represented 4.94 ± 0.66% of the ingested [^{3}H]-DON, being statistically lower than the percentage found

in the tissues (17.74 ± 6.71, p=0.001). No differences (p>0.05) were observed during the 24 h period regarding the presence of [³H]-DON in kidney, liver and skin of the trout juveniles, which remained relatively low. GIT showed a relative constant amount of [³H]-DON during the first six hours, decreasing after this to a final amount of 10.02 ±10.45 ng DON at twenty-four hours. No statistical differences were found for this tissue for the twenty-four hours experimental period.

After twelve and twenty-four hours, the percentage of DON present in water (63.50 ± 27.76% and 62.15 ± 35.56%, respectively) compared with the total tube-fed DON (125±0.019 ng DON), was significantly higher (p=0.001) than in previous sampling points (1 h=4.94 ± 0.66, 3 h=16.62 ± 12.80 and 6 h=11.03 ± 6.09%). The total recovery of [³H]-DON also increased significantly at twelve and twenty-four hours sampling (69.86 and 75.22%, respectively).

At the end of the experimental period (24 h of being tube-fed) trout's presented marginal amounts of [³H]-DON in the tissues (GIT=10.02 ± 10.45 ng; liver=0.87 ± 0.85 ng; muscle=3.61 ± 2.84 ng; kidney=0.57 ± 0.46 ng and skin=1.58 ± 1.57 ng). At this time, most of the [³H]-DON was found in the water (77.84 ± 44.54 ng), representing 62.15 ± 35.56% of the initial tube-fed DON (125 ± 0.019 ng DON). The period between six and twelve hours after tube-feeding seems to be the turning point where it is possible to observe a higher level of [³H]-DON being excreted into the water compared to levels of [³H]-DON in the fish (Figure 1). After twenty-four hours, the sum of [³H]-DON in all tissue samples showed a total of 16.37 ± 14.46 ng of ³H-DON compared to 77.84 ± 44.54 ng ³H-DON excreted into the water (Figure 1).

Determination of toxicokinetic parameters

The toxicokinetic parameters of [³H]-DON in tube-fed rainbow trout are presented in Table 2. The distribution and excretion profiles differed depending on the tissue. GIT presented the highest concentration of [³H]-DON (C_{max}=65.28 ng g $^{-1}$) after 3 h of tube feeding (t_{max}=3 h). This maximum concentration decreased thereafter with a half-life ($t_{1/2}$) of 88.51 h (four time points considered, r^2=0.706). Samples from the liver showed a lower peak concentration of [³H]-DON (C_{max}=12.91 ng g $^{-1}$), also at 3 h after tube feeding (t_{max}=3 h) however, with a $t_{1/2}$ of 95.14 h (four time points considered, r^2=0.444). Toxicokinetics for kidney, muscle and skin were not calculated.

Water/ tissues	Sampling points									
	1 hour		3 hours		6 hours		12 hours		24 hours	
	DON (ng)	% of DON relative to ingested DON	DON (ng)	% DON relative to ingested DON	DON (ng)	% DON relative to ingested DON	DON (ng)	% DON relative to ingested DON	DON (ng)	% DON relative to ingested DON
Water	6.19 ± 0.83^{c}	4.94±0.66^{a}	20.81 ± 16.04^{bc}	16.62 ± 12.80^{a}	16.58 ± 3.93^{c}	11.03 ± 6.09^{a}	63.50 ± 27.76^{a}	50.71 ± 22.17^{b}	77.84 ± 44.54^{a}	62.15 ± 35.56^{b}
GIT	20.56 ± 8.30		18.27 ± 1.88		21.79 ± 7.95		13.14 ± 6.14		10.02 ± 10.45	
Liver	1.44 ± 0.67		0.93 ± 0.17		1.24 ± 0.54		1.14 ± 0.55		0.87 ± 0.85	
Muscle	n.s.	17.74±6.71^{b}	6.14 ± 2.48^{ab}	22.30 ± 3.48^{b}	8.89 ± 4.00^{a}	28.80 ± 8.54^{a}	6.67 ± 2.30^{ab}	19.15 ± 7.70^{a}	3.61 ± 2.84^{b}	13.07 ± 11.54^{a}
Kidney	0.23 ± 0.13		0.30 ± 0.20		0.52 ± 0.41		0.31 ± 0.08		0.57 ± 0.46	
Skin	n.s.		2.44 ± 1.35		3.62 ± 2.30		2.78 ± 1.14		1.58 ± 1.57	
[³H]-DON Recovery	--	22.68%	--	38.92%	--	39.83%	---	69.86%	--	75.22%

Values are means ± S.D. for each sampled tissue and water. Different letters indicate statistically significant differences (p<0.05, one-way ANOVA) between time points for tissues and water. Kidney, liver, GIT and skin did not show significate differences during the 24-hour period. For the percentage of DON relative to ingested DON different letters indicate statistically significant differences (p<0.05, one-way ANOVA) within same sampling time point between water and sum of tissue. n.s. = not sampled.

Table 1: Deoxynivalenol (DON distribution in fish tissue (ng of DON; gastro-intestinal tract, liver, kidney, muscle, kidney and skin) and in water, after tube-feeding pellets labelled with [³H]-DON, for each sampling points (1 to 24 hours). And percentage of DON in tissues (sum of tissues per sampling point) or in water relatively to tube-fed amount (125 ± 0.019 ng) DON.

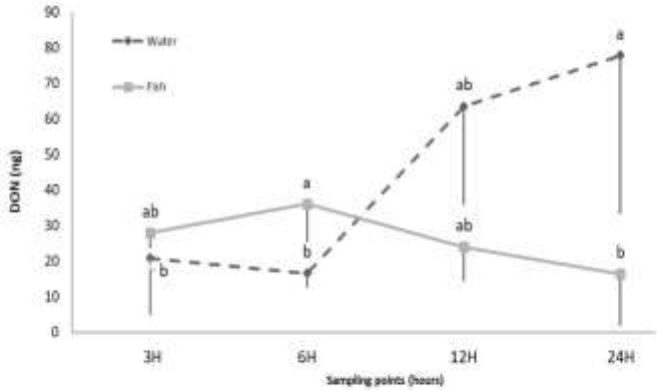

Figure 1: Deoxynivalenol (DON) in fish tissue (sum of total DON detected in the gastro-intestinal tract, liver, kidney, muscle and skin) and excretion into water at 3 h, 6 h, 12 h and 24 h after tube-feeding a meal labelled with 3H-DON. Values are means ± S.D. Different letters within each compartment indicate statistically significant differences (p<0.05, one-way ANOVA) between sampling points.

Parameters	GIT	Liver
Interception	1.80	1.08
Slop	-0.003	-0.003
C_{max}	65.28	12.91
t_{max}	3	3
C_0	63.08	12.26
K_{el}	0.008	0.007
$t_{1/2}$	88.51	95.14

Concentration at time zero (C_0; ng g^{-1} DON) was determined from the tissue concentration–time curves obtained. Elimination constants (K_{el}; h) were determined by curve regression ($C(t)=c0*e(K_{el}*t)$). The elimination half-life was calculated as in $t_{1/2}=\ln 2/K_{el}$. C_{max} (ng g^{-1} DON) is the peak concentration of [^{3}H]-DON after administration at a certain time (t_{max}, h). Toxicokinetics for muscle and skin were not calculated.

Table 2: Toxicokinetic parameters of [^{3}H]-DON tube-fed to rainbow trout.

Deoxynivalenol metabolization assay

We did not detect any DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) in the water samples taken after 24 h from the chambers of the tube-fed animals that received 663 ng of DON each (limits of detection: 0.2 µg kg^{-1} for DOM-1, DON-3-sulfate and DOM-3-sulfate)., We detected 0.08 ± 0.063 ng of DON per ml of water. The recovery of DON represents 24.13% of the total amount fed via a tube to the animal.

Discussion

Understanding the biological fate of DON in aquaculture species is of vital importance, as it sheds light on the carryover of DON into edible tissues and on possible adverse effects of the toxin on the animal. Knowledge of the biological fate of DON also provides understanding of how to address and mitigate the impact of DON in the animal, for example by developing a DON-detoxifying feed additive. Detoxifying feed additives are intended to decrease the bioavailability of the toxin to the animal in the digestive tract, reducing any possible negative effect of the toxin on the GIT and its absorption into the blood. Knowledge about retention time of DON in digesta, absorption, and distribution, as well as possible biodegradation of DON by the indigenous GIT bacteria are fundamental to the development of a DON-detoxifying strategy. In the current trial, [^{3}H]-DON was detected in all sampled tissues (GIT, liver, muscle, kidney and skin). One hour after tube-feeding, [^{3}H]-DON was detected in the liver of the fish, indicating that DON absorption is relatively fast in rainbow trout juveniles. Due to technical challenges, it was not possible to collect plasma for analysis. Analysis of DON levels in plasma could have confirmed that DON was absorbed quickly. Bernhoft et al. [10] reported that the concentration of DON found in *Salmo salar* liver samples reached a maximum concentration one hour after intake. In the present study for rainbow trout, the maximum concentration was only achieved after 3 h and half-life was higher ($t_{1/2liver}$ 95.14 h) than reported by Bernhoft et al. [10] ($t_{1/2liver}$ 6h), which might explain the higher sensitivity of trout to DON [15]. Bernhoft et al. [10] obtained a maximum DON plasma concentration at time zero and $t_{1/2\ plasma}$ 15.1 h, showing that DON is rapidly absorbed in salmon. Moreover, the low $t_{1/2\ plasma}$ reported by Bernhoft et al. [10] indicates the possibility of some absorption of DON from the stomach. A rapid absorption of DON was also observed by Dänicke et al. [16] and Eriksen et al. [17] in pigs. Focusing on the first hour after tube-feeding, [^{3}H]-DON was detected mainly in the GIT (20.56 ± 8.30 ng). However, some radioactivity was also detected in the water (equivalent to 6.19 ± 0.83 ng [^{3}H]-DON). As regurgitation can be excluded (visual confirmation), any [^{3}H]-DON detected in the water was excreted and not vomited or leached from the pellets. The low passage time of [^{3}H]-DON through the GIT (< 1 hour), which was lower than the trout average gastric emptying time (> 6 h; depending on temperature and meal type and size; see Langton [18], could be due to the high-water solubility of DON and excretion of DON with the fluid phase of the chyme. Accordingly, Dänicke et al. [16]. (2004) reported that in pigs, DON leached from pellets into the liquid phase in the stomach and was emptied with the liquid phase of the chime and faster than the solid phase of the chyme [19]. While the rapid excretion of DON may prevent immediate negative effects of dietary DON on the GIT of the trout, the high solubility and stability of DON in water may lead to re-ingestion by the fish.

The tube-feeding technique was selected to simulate a normal pellet intake, eliminating the risks of DON leaching from the pellets and ensuring the intake of a defined amount of DON. While the employed experimental setup revealed the rapid passage of [^{3}H]-DON through the GIT, which is an important and novel information, it was associated to some technical challenges in the methodology used. The recovery of [^{3}H]-DON, especially during the first three sampling time points (1, 3 and 6 hours) was relatively low (22.68%, 38.92% and 39.83%, respectively). Despite the metabolic chambers being a closed system, some losses were expected due to sampling limitations. For instance, DON residues in the head and skeleton were not analysed and DON residues in the blood could not be analysed due to coagulation during anaesthetic overdose euthanasia. This inevitably contributed to losses in the [^{3}H]-DON budget. Indeed, *Salmo salar* brain was shown to absorb DON [10]. Arguably, the most important factor that contributed to the low recovery of [^{3}H]-DON was the loss of material from the GIT during sampling. While most of the solid phase of the chyme and the faeces remained in the GIT during tissues sampling, the fluid phase of the chyme was probably lost during the sampling procedure. Consequently, a loss of DON contained in the fluid phase may have contributed to the recovery of constantly low [^{3}H]-DON levels from the GIT at the first sampling time points (1 to 6 h). At twelve and twenty-four hours, [^{3}H]-DON recovery was higher, namely 69.86% and 75.22% respectively. At these time points recoveries was probably mostly influenced by tissues not collected (head, blood, skeleton with muscle attached) as digestion had already happened.

The low absorption of DON during the first 1, 3 and 6 hours (17.74 ± 6.71; 22.30 ± 3.48; 28.80 ± 8.54% in relation to tube-fed DON amount, respectively) may also be explained by the trouts' physiological condition prior to the study. In the present trial, trout were fed a non-contaminated diet (for three weeks) prior to the [^{3}H]-DON tube feeding. It has been reported that chronic exposure to DON might cause the destruction of tight junctions [20,21] leading to increased DON absorption. We assume that in the present study, the trouts' physiological conditions due to the three weeks acclimation were optimal and intestinal barrier would not be much impacted by the short period of DON exposition (maximum of twenty-four hours). For future studies, it would be interesting to adapt the experimental procedures for the tube-feeding technique in order to enable us to collect the fluid phase of the chyme from the GIT during sampling and to determine DON residues in the fluid phase, especially during the first six hours of sampling (maximum expected digestion time). Furthermore, due to a possible harmful effect of chronic DON exposure on the intestinal barrier it would be relevant to assess the toxicokinetics of DON in trout chronically exposed to DON before the toxicokinetic experiment. The low excretion of DON into the water during the first six hours after DON tube-feeding indicates a long DON retention time that increases the probability of DON absorption and of a negative effect of DON on the GIT The period between six and twelve hours

seems to be the turning point in terms of DON excretion. Twelve hours after tube-feeding, the trout excreted 50.71 ± 22.17% of the tube-fed DON amount, while on the previous sampling point (six hours) the trout excreted only 11.03 ± 6.09%. Taking into account a digestion time of six hours at 15 °C (Langton 1977) [16] it can be expected that most of the DON was retained in the GIT and excreted after digestion.

In a previous study, our group found that DON is metabolised to DON-3-sulfate in trout [22]. In this previous study, more than 80% of the mycotoxin recovered from faeces was DON-3-sulfate. The location of the formation, absorption and elimination of DON-3-sulfate has not been identified, but evidence suggested that DON might be metabolised into DON-3-sulfate in the intestinal mucosa [23]. In the present trial, only DON was found in the water from metabolic chambers and no DON-3-sulfate was detected. Based on this discrepancy, it is tempting to speculate that the conversion of DON to DON-3-sulfate is catalysed by the gut microbiota and that its incidence depends on gut microbial community composition. As there were no DON metabolites detected, the detected radioactivity likely originated from intact DON molecules. However, unknown DON metabolites may have been missed. The low concentrations of DON measured in this trial were near the limit of detection of the analysis method (0.2 μg kg^{-1}). This might have contributed to the low recovery of non-radiolabelled DON.

Conclusion

Despite some limitations of the experimental procedures, which influenced the obtained results, especially during the first three sampling points, we could conclude that one hour after tube-feeding, [^{3}H]-DON was detected in the liver samples of fish, indicating a rapid absorption of DON. In the first hour, [^{3}H]-DON was present in the GIT (20.56 ± 8.30 ng). However, 6.19 ± 0.83 ng was also detected in the water at this sampling point. The fast excretion of [^{3}H]-DON (faster than the average trout gastric emptying time) suggests that DON, as a water-soluble compound, is excreted with the liquid phase of the chyme. The presence of [^{3}H]-DON in the GIT was stable during the first six hours. This long residence time of DON in the GIT may compromise the health of the GIT and favour absorption. Our data suggests that an effective DON detoxifying method should have a period of action of ≤ 6 h. Furthermore, as most of the excretion can be expected to happen after six hours, the detoxification should be irreversible at GIT conditions.

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Declaration of interest, funding source and author Contributions

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Oral presentations in conferences

1. **Rui A. Gonçalves** Occurrence of Mycotoxins in Feed and Food: A Real Risk to Aquaculture? Aquafeed Platform EUROPE 2016. 19th Practical Short Course: Trends and Markets in Aquaculture Feed Ingredients, Nutrition, Formulation and Optimized Feed Production and Quality Management. Novotel Gent Centrum, Ghent, Belgium - 3 & 4 December **2018**
2. **Rui A. Gonçalves**, Carmen Navarro-Guillén, Neda Gilannejad, Jorge Dias, Dian Schatzmayr, Gerlinde Bichl, Tibor Czabany, Francisco Javier Moyano, Manuel Yúfera, Simon Mackenzie, Gonzalo Martínez-Rodríguez. IMPACT OF DEOXYNIVALENOL ON RAINBOW TROUT: GROWTH PERFORMANCE, DIGESTIBILITY, KEY GENE EXPRESSION REGULATION AND METABOLISATION. ASIAN-PACIFIC AQUACULTURE 2018. April 23-26, **2018**. Taipei, Taiwan.
3. **Rui A. Gonçalves**, Ursula Hofstetter, Dian Schatzmayr, Timothy Jenkins. MYCOTOXINS IN SOUTHEAST ASIAN AQUACULTURE: PLANT-BASED MEALS AND FINISHED FEEDS. ASIAN-PACIFIC AQUACULTURE 2018. April 23-26, **2018**. Taipei, Taiwan.
4. Michele Muccio, **Rui A. Gonçalves**, Ursula Hofstetter, Timothy Jenkins, Dian Schatzmayr. WORLDWIDE MYCOTOXIN OCCURRENCE IN PLANT MEALS: AN OVERLOOKED RISK TO AQUACULTURE? ASIAN-PACIFIC AQUACULTURE 2018. April 23-26, 2018. Taipei, Taiwan.
5. **Rui A. Gonçalves**. Mycotoxins: The Hidden Threat. AquaEx India **2018**, 15 – 17 March, Hyderabad, India.
6. **Rui A. Gonçalves**. Mycotoxins in aquaculture: occurrence and impact in rainbow trout (*Oncorhynchus mykiss*). AQUAFEED HORIZONS 2017, Cologne Exhibition Halls, (Koelnmesse), Cologne, Germany, June 14, **2017**.
7. **Rui A. Gonçalves**. MYCOTOXINS IN AQUACULTURE: AN OVERLOOKED RISK?, CCMAR SEMINARS, Centre of Marine Sciences, University of Algarve, Portugal, 15 March **2017**
8. **Rui A. Gonçalves**, Simon Menanteau-Ledouble, Dian Schatzmayr, Mansour El-Matbouli. THE IMPACT OF DEOXYNIVALENOL IN RAINBOW TROUT (Oncorhynchus mykiss). Aquaculture Europe 17 – Cooperation for Growth, October 17-20, **2017**, Dubrovnik, Croatia.

9. S. Menanteau-Ledouble, M. Scholler, A. Eder, **R. Gonçalves**, G. Santos, M. El-Matbouli**.** Effect of mycotoxin contamination on the rainbow trout Oncorhynchus mykiss. 18th International Conference on Diseases of Fish and Shellfish. 4-8 September **2017**, Belfast, United Kingdom.

10. **Rui A. Gonçalves**, Dian Schatzmayr, Michele Muccio, João Sendão. SENSITIVITY OF *Litopenaeus vannamei* TO *Fusarium* MYCOTOXINS: THE TOXICITY OF LOW DOSE OF FUMONISINS AND DEOXYNIVALENOL AND ITS ACCUMULATION IN CLOSED SYSTEMS. ASIAN-PACIFIC AQUACULTURE **2017**. 24-27 July, 2017, Kuala Lumpur, Malaysia.

11. **Rui A. Gonçalves**, Ursula Hofstetter, Dian Schatzmayr. MYCOTOXINS IN SE ASIA AQUACULTURE: A NEGLECTED THREAT? ASIAN-PACIFIC AQUACULTURE **2017**. 24-27 July, 2017, Kuala Lumpur, Malaysia.

12. **Rui A. Gonçalves**, Ursula Hofstetter, Dian Schatzmayr. MYCOTOXINS IN SE ASIA AQUACULTURE PLANT-BASED MEALS. ASIAN-PACIFIC AQUACULTURE **2017**. 24-27 July, 2017, Kuala Lumpur, Malaysia. – Best PhD student abstract –

Poster presentations in congresses

1. **Rui A. Gonçalves**, Sofia Engrola, Cláudia Aragão, Simon Mackenzie, Gerlinde Bichl, Tibor Czabany, Dian Schatzmayr. Fate of [^{3}H]-deoxynivalenol in rainbow trout (Oncorhynchus mykiss) juveniles-Tissue distribution and excretion. 3rd Aquaculture Conference 2018. September 25-28, **2018**. Qingdao, China.
2. **Rui A. Gonçalves**, Carmen Navarro-Guillén, Neda Gilannejad, Jorge Dias, Dian Schatzmayr, Gerlinde Bichl, Tibor Czabany, Francisco Javier Moyano, Manuel Yúfera, Simon Mackenzie, Gonzalo Martínez-Rodríguez. Impact of deoxynivalenol on rainbow trout: Digestibility and metabolism. 3rd Aquaculture Conference 2018. September 25-28, **2018**. Qingdao, China
3. M. Muccio, **R.A. Gonçalves**, T. Jenkins, U. Hofstetter, D. Schatzmayr. Worldwide mycotoxin occurrence in plant meals: An overlooked risk to aquaculture? 3rd Aquaculture Conference 2018. September 25-28, **2018**. Qingdao, China.
4. **Rui A. Gonçalves**, Sofia Engrola, Cláudia Aragão, Simon Mackenzie, Gerlinde Bichl, Tibor Czabany, Dian Schatzmayr. FATE OF [^{3}H]-DEOXYNIVALENOL IN RAINBOW TROUT (Oncorhynchus mykiss) JUVENILES: TISSUE DISTRIBUTION AND EXCRETION. ASIAN-PACIFIC AQUACULTURE 2018. April 23-26, **2018**. Taipei, Taiwan.
5. **Rui A. Gonçalves**, Michele Muccio, Ursula Hofstetter, Dian Schatzmayr. WORLDWIDE MYCOTOXIN OCCURRENCE IN PLANT MEALS: A REAL RISK TO AQUACULTURE DEVELOPMENT? Aquaculture Europe 17 – Cooperation for Growth, October 17-20, **2017**, Dubrovnik, Croatia. – Best PhD Student Poster -
6. **Rui Alexandre Gonçalves**, Marco Tarasco, Dian Schatzmayr, Paulo Gavaia. MONILIFORMIN: AN EMERGING THREAT TO AQUACULTURE SPECIES? Aquaculture Europe 17 – Cooperation for Growth, October 17-20, **2017**, Dubrovnik, Croatia.

Awards during PhD course

1. **Ibrahim Okumus Award for best poster by European Aquaculture Society** students group at Aquaculture Europe 17 – Cooperation for growth, for the poster "WORLDWIDE MYCOTOXIN OCCURRENCE IN PLANT MEALS: A REAL RISK TO AQUACULTURE DEVELOPMENT?".
2. **WAS-APC Student Award** at ASIAN PACIFIC AQUACULTURE 2017 for best abstract "Mycotoxins in SE Asia aquaculture plant-based meals"