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Feed contamination with zearalenone promotes growth but affects the immune system of rainbow trout

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ABSTRACT

To investigate the effects of feed contamination with zearalenone (ZEN) at the current European Commission (EC) guidance value (2 mg·kg^{-1} feed) on the growth and health of rainbow trout, we performed a long-term feeding trial under aquaculture conditions. It started with the external feeding of the fish larvae, and continued for 96 weeks, at which point the fish had reached market size. To assess the growth of fish and their feeding efficiency throughout this period, the fish were regularly weighed and measured, and their feed consumption was monitored. Additionally, to investigate potential health effects, after 72 weeks of the exposure to ZEN, the fishes' blood was analyzed for major hematological and biochemical indices, and their head kidney, spleen, and liver were examined for morphological, histopathological, cytological, and molecular changes. Finally, to gain insight into the metabolism and distribution of ZEN in fish, the content of free and glucuronidated forms of ZEN and its major metabolites was measured in the intestine, liver, and muscles of the exposed fish. The feed-borne exposure of rainbow trout to ZEN at a dose of 2 mg·kg^{-1} feed resulted in higher feeding efficiency and growth rate, most probably due to the anabolic properties of the ZEN metabolite. Importantly for the consumers of fish, despite absorption and metabolism of ZEN in the digestive system of the fish that had been exposed for 72 weeks, the residuals of ZEN were not transferred to the fishes' muscles, which rules out a potential risk to human health related to the consumption of fish meat. However, the increased growth of fish fed with the contaminated feed may come at some cost, as the exposure to ZEN was associated with modulation of key components of the adaptive and innate immune systems. Moreover, the trunk kidney of ZEN-fed fish showed massive inflammation that was likely caused by pathogen infection. These findings raise concerns about fish health under the current recommended EC guidance values.

1. Introduction

Zearalenone (ZEN) is a mycotoxin produced by some species of *Fusarium* molds that can be frequently found in plant material, including cereals and legumes. These toxicogenic fungi infect agricultural crops, which results in worldwide contamination of food products and animal feeds with ZEN and other mycotoxins [19,49,67]. Due to its adverse effects in animals, this mycotoxin is widely considered as an undesirable feed substance and it has been a matter of ongoing health concerns and risk assessments for decades [16,30].

The most prominent effects of ZEN toxicity involve structural disorders and dysfunction in the reproductive system, which have been extensively described in livestock animals like swine, cattle, or poultry [36,67]. Due to its well-characterized binding affinity to estrogen receptors, ZEN mimics the action of steroid hormones (i.e. estrogens), which can lead to reproductive disorders in exposed livestock animals, including decreased libido, disrupted ovulation, and even infertility [17,30,34]. Importantly for research on aquatic animals, it has been

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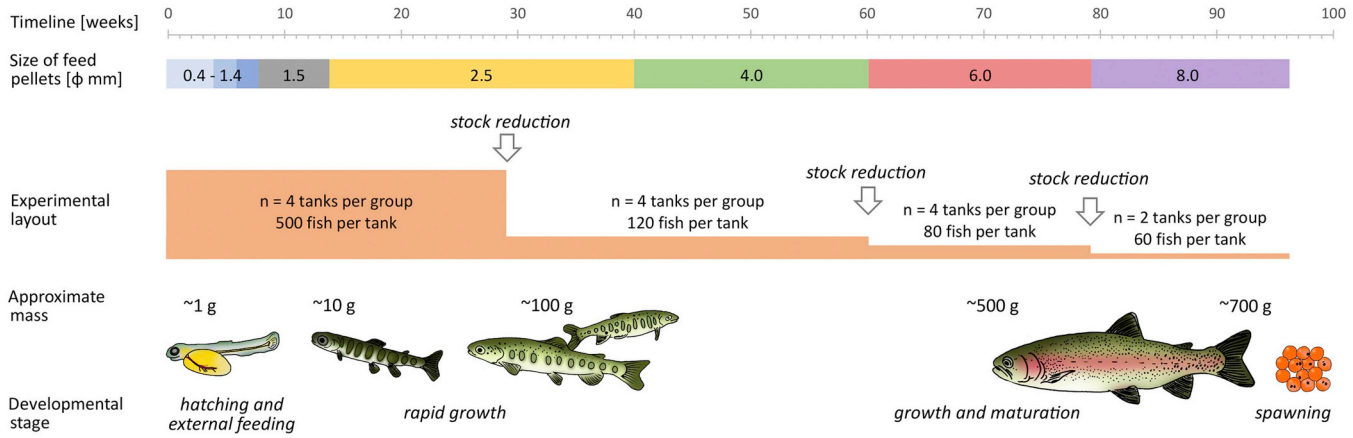

Fig. 1. Design of the entire project, which examined the life-cycle effects of rainbow trout exposure to zearalenone in feed at the European Commission's guidance value of 2 mg·kg^{-1} feed (please visit http://bit.ly/fish-zen for more information about the project). In the study reported here, which is a part of the project, we focused on growth and health-related effects.

shown that ZEN may also affect reproduction of fish [2,8,51,52,65]. Moreover, in addition to reproductive disorders, there is a growing conviction that the estrogenic properties of ZEN may also account for the immunomodulatory effects that have been reported in a number of *in vivo* and *in vitro* studies in mammals (e.g. Refs. [5,10,26,32,47]). However, possible immunomodulatory effects of ZEN in fish have been neglected up to this point, except for the studies of Pietsch et al. [42–44] on common carp, *Cyprinus carpio*.

In swine, cattle and poultry, the uptake, metabolism and excretion of ZEN have been extensively studied. After ingestion with contaminated feed, ZEN is rapidly absorbed from the gut and primarily transformed in the intestine and liver into its two major metabolites, α- and β-zearalenol (α- and β-ZEL), by α- and β-hydroxysteroid dehydrogenase, respectively. These metabolites can be further reduced into α- and β-zearalanol (α- and β-ZAL, which are also termed zeranol and taleranol, respectively). In the next phase, the metabolites of ZEN are conjugated, mainly with glucuronic acid by UDP-glucuronyl transferases, and then excreted in bile and urine [13,17]. Studies that have investigated piscine metabolism of the mycotoxin suggest that the major biotransformation pathways of ZEN in fish are similar to those described in mammals [31,45,64]. However, a gap in our knowledge persists regarding the specific roles/activities of these metabolites in the response of fish to treatment with the parent compound.

The rapid development of aquaculture has increased the demand for raw materials that are used to produce feeds. Due to decreasing supplies and increasing costs of fishmeal and fish oil, the industry has been forced to search for alternative sources of nutrients, such as plant materials, which constitute a growing source of dietary proteins and lipids for farmed salmonid fish, like trout or salmon [57]. However, the use of plant additives in modern aquaculture raises a concern about potential health issues related to the presence of mycotoxins in fish feeds [1,20,33]. Indeed, numerous reports testify to the presence of ZEN in feed materials and feeds for fish (e.g. Refs. [19–21,38,66]). For example, ZEN was found in all fish-feed samples collected from Central Europe by Pietsch et al. [46]; with content up to 0.511 mg·kg^{-1} feed, and in those collected from Southeast Asia by Gonçalves et al. [19]; with content up to 0.153 mg·kg^{-1} feed. The contamination of fish feeds with mycotoxins may perturb the health of fish, and thus reduce the profitability of aquaculture, e.g. due to increased fish mortality or disease-treatment costs. Moreover, if the mycotoxins are transferred into the meat of farmed fish, the contaminated feed may pose an additional health risk to the consumers of the aquaculture products. Thus, knowledge of the toxicokinetics and the biological effects of ZEN is necessary to improve our understanding of the potential threats resulting from plant-based feeding solutions in sustainable aquaculture.

According to the current recommendation of the European Commission (EC), the content of ZEN in animal feed and feedstuff should not exceed 2 mg·kg^{-1}, with the exception of maize and its by-products [14]. This EC guidance value was established based on the European Food Safety Agency's scientific opinion [12], which was given without reference to any specific data on the biological effects of ZEN in fish or the mycotoxin's presence in fish feeds. Even though such information has been published in recent years [19–21,38,43,44,46,64–66], to date, there has been no systematic research comprehensively evaluating how the presence of ZEN in feed at the EC guidance value affects the growth and health of economically-important fish species throughout their life-cycle. Such information would provide a strong basis for evaluating the safety of the current guidance value [14].

Therefore, we decided to investigate how exposure to ZEN at the EC guidance value (2 mg·kg^{-1} feed) would affect the growth performance and health of rainbow trout. Exposure began at the start of external feeding, and continued for 96 weeks, at which point, the fish had reached market size. To assess the growth of fish and their feeding efficiency, the fish were regularly weighed and measured, and their feed consumption was monitored. To investigate the health-related effects of exposure to ZEN, after 72 weeks of exposure, blood was analyzed for hematological and biochemical indices, and selected organs (i.e. head kidney, spleen, and liver) were examined for morphological, histopathological, and cytological changes. Furthermore, molecular methods were used to assess the immune response of the fish. Finally, to gain insight into the metabolism and distribution of ZEN in fish tissues, the content of free and glucuronidated forms of ZEN and its major metabolites was measured in the intestine, the liver, and the white muscles of the exposed fish.

2. Materials and methods

This study is a part of larger project which aimed to examine the life-cycle effects of rainbow trout exposure to ZEN at the European Commission's guidance value of 2 mg·kg^{-1} feed. This life-cycle feeding trial began with the start of external feeding of the fish larvae and continued for 96 weeks (Fig. 1). Whereas this study focuses on growth performance and health of the fish up to the point when they reach market size, forthcoming studies will be focused on other issues, including reproductive or transgenerational effects of the life-cycle exposure.

2.1. Experimental design of the project

To study the effects of feed contamination with ZEN on male and female fish, rainbow trout larvae were obtained by spawning natural males and females. Thus, the offspring population contained individuals of both sexes (approximately 50% males and 50% females). Swimming larvae were divided into two experimental groups that were reared on either commercial (blank) feed (control group) or ZEN-contaminated feed (exposed group). This dietary treatment was applied from the first day of external feeding and lasted for 96 weeks. Initially, the fish were separated into 4 tanks per group with n = 500 fish in each tank. However, due to economic reasons and the increasing amount of labour needed for feed preparation and stock maintenance as the fish grew, the number of fish was reduced at 29, 60, and 79 weeks of the experiment. At the final reduction step (i.e. 79 weeks), n = 120 fish from each group were microchipped, and to reduce stress associated with decreased stock density, the number of tanks per group was reduced to two. To ensure that the fish number in each tank was reduced in a random way, all fish from a tank were first crowded in a minimal volume of water and then thoroughly mixed just before separating a representative group of fish for the following experimental period. These reduction steps coincided with detailed biometric measurements or collection of samples for selected analyses (Fig. 1).

2.2. Contamination of the fish feed

For experimental feeding of the fish throughout their life-cycle, we used a commercially available premium fish feed designed for trout feeding. Before use, each batch of the purchased trout feed pellets was analyzed for the presence of background contamination with ZEN and other mycotoxins (aflatoxin B_1, deoxynivalenol, ochratoxin A). The analyzed feed then either served as blank (non-contaminated) feed for the control group of fish, or was used to prepare contaminated batches of feed for the exposed group of fish, according to a previously described method [65]. Briefly, an analytical sample weight of ZEN (Cayman Chem.; USA) was dissolved in 96% ethanol, then atomized onto a single layer of the feed pellets, and left to evaporate. After drying, the layer of feed was mixed, once again sprayed with the solution of ZEN, and left for drying. Once dry, the pellets were collected in a larger batch of contaminated feed (usually 25 kg) and then thoroughly mixed. The actual content of ZEN in each batch of contaminated feed was determined using ZearalaTest™ immunoaffinitive columns combined with HPLC (see below for methodological details). Major nutritional characteristics and amounts of the feed used in this study, as well as content of the mycotoxins in both the control and the contaminated batches are presented in Table 1. The actual contents of ZEN measured in the feed were used to calculate the average content that the fish were exposed to throughout the experimental feeding trial, according to the equation:

$$\textit{mean ZEN content}\ [\mathrm{mg \cdot kg^{-1}}] = \frac{\textit{sum of masses of ZEN in each feed fraction}\ [\mathrm{mg}]}{\textit{sum of total masses of each feed fraction consumed}\ [\mathrm{kg}]}$$

2.3. Fish maintenance and exposure

Fish breeding, maintenance, and exposure were conducted at the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn; Poland). The institute is certified by the official veterinary inspection service (ID No. 22 05 92 01; Powiatowy Inspektorat Weterynarii w Kartuzach; Poland). Thus, the broodstock is routinely tested for the presence of bacterial and viral fish pathogens; the fish were found to be free of *Renibacterium salmoninarium* causing Bacterial Kidney Disease, or viruses causing Viral Hemorrhagic Septicemia and Infectious Hematopoietic Necrosis. All fish were housed and handled in compliance with widely accepted guidelines for laboratory animal care (according to the directive No. 2010/63/EU of the European Parliament and of the Council). The experiment was approved by the Local Ethical Commission in Olsztyn, Poland (resolution No. 74/2014 issued on the 10th December 2014). Throughout the whole experiment, all fish were kept in tanks of a flow-through hatchery system supplied with surface water, and the fish were reared under natural photoperiod and in natural (ambient) water temperatures.

Throughout the feeding trial, the fish were intensively fed with automatic belt feeders. The daily amount of feed given was calculated based on the water temperature, the caloric content of the feed, and the predicted fish mass (according to [18]). The size of the feed ration for each tank was calculated separately. In order to monitor the actual mass and correct the predictions of fish growth, all fish from each tank were collectively weighted at the hatchery every 2–3 weeks. The fish were deprived of the feed usually 1 or 2 days before collection of samples. Each day, the tanks were inspected for dead fish and unconsumed feed. Temperature and oxygen concentration of the system water that was used to supply the fish tanks were also routinely measured. Every six months, major chemical indices of water quality were also determined. The details of the fishes' maintenance and exposure, and the results of the water quality analysis are presented in Supplement 1 and 2.

It has been shown that ZEN may naturally occur in surface and groundwaters [24]. To exclude system water from our hatchery as a possible source of contamination with ZEN, samples of the water from the influent of the fish tanks were collected every month of the experiment for the HPLC analysis.

2.3.1. Survival analysis

To assess the significance of differences in time to death between tanks and between groups, survival analysis based on the Kaplan-Meier method was used. In this analysis, “event” refers to the observed death of an animal, and “censored” refers to the individuals removed from the tanks because of reduction in the number of fish in the study (see section 2.1) or for sample collection, or that did not die at the end of the experiment. The analysis was performed using SPSS Statistics 24 (IBM; USA), and differences were considered to be significant at $p < 0.05$.

2.4. Collection of samples

In order to obtain insight into the health status of the fish, after 72 weeks of exposure, 36 randomly selected individuals from each group were anesthetized and weighed. Then, 12 fish from each group were euthanized and frozen (−20 °C) in case further analyses and observations would be needed, while another 24 from each group were measured, examined and sampled as follows. Blood samples were taken from the caudal vein of the anesthetized fish and were immediately processed for direct blood measurements and plasma isolation, according to a previously described procedure [65]. Then, the fish were euthanized, and their body cavity was opened for visual inspection. The liver with gall bladder was removed, weighed, and then fragmented and preserved for histological and molecular analyses. After all the internal organs were removed from each inspected individual, the gutted carcass (with head) was weighed. Next, fragments of the head kidney and the spleen, or other macroscopically-changed tissues were preserved for further histological or molecular evaluation. Finally, the rest of the liver (with the gall bladder), the dorsal part of the white muscles, and the caudal part of the intestine with its content (e.g an ~8 cm section, starting from the anus) were stored at −20 °C for further measurement of the content of ZEN residuals (*HPLC analysis*).

2.5. Feed conversion rate (FCR) and thermal growth coefficient (TGC)

The FCR and the TGC were calculated based on data acquired at the rearing facility, according to the equations:

Table 1
Major constituents and concentration of selected mycotoxins in the fish feed used in the 96-week experiment aiming to assess the life-cycle effects of rainbow trout exposure to zearalenone at a dose of 2 mg·kg^{-1} feed (please also refer to Fig. 1 and Supplement 1 for more details).

Pellet size [mm]	Major components [%] and Digestive energy [MJ·kg^{-1}]		Dates and feeding period	Mass of contaminated feed consumed only by the exposed group [kg]	Mean content ± S.D. [μg·kg^{-1}]			
					Contamination with ZEN in feed for the exposed group		Background contamination in blank (control) feed	
0.4–0.7	Protein	55	5 May – 1 June	0.9	ZEN	1924.0	ZEN	99.0
	Fat	16	2015			± 23.3	DON	n.d.
	Sugars	8	(0–4 weeks)		(3 samples from 1 batch)		OTA	10.6
	Energy	19.0					AFB1	n.d.
0.6–1.0	Protein	54	2–15 June 2015	1.2	ZEN	2037.3	ZEN	58.0
	Fat	18	(5–6 weeks)			± 73.3	DON	n.d.
	Sugars	8			(3 samples from 1 batch)		OTA	9.4
	Energy	19.5					AFB1	n.d.
0.8–1.4	Protein	54	16 June – 6 July	3.4	ZEN	1965.0	ZEN	60.0
	Fat	18	2015			± 40.6	DON	n.d.
	Sugars	8	(7–9 weeks)		(3 samples from 1 batch)		OTA	10.1
	Energy	19.5					AFB1	n.d.
1.5	Protein	52	7 July – 12 Aug	13.9	ZEN	1876.0	ZEN	96.5
	Fat	20	2015			± 84.6	DON	n.d.
	Sugars	8.5	(10–14 weeks)		(3 samples from 1 batch)		OTA	9.2
	Energy	19.9					AFB1	n.d.
2.5	Protein	44	13 Aug 2015–7	96.8	ZEN	2009.9	ZEN	15.0
	Fat	25	Feb 2016			± 145.9	DON	27.1
	Sugars	15.5	(15–40 weeks)		(12 samples from 6 batches)		OTA	10.6
	Energy	21.0					AFB1	n.d.
4	Protein	43	8 Feb – 28 June	81.9	ZEN	2012.5	ZEN	n.d.
	Fat	26	2016			± 166.1	DON	38.4
	Sugars	15.5	(41–60 weeks)		(8 samples from 4 batches)		OTA	n.d.
	Energy	21.1					AFB1	n.d.
6	Protein	41	29 June – 4 Nov	150.0	ZEN	2080.8	ZEN	n.d.
	Fat	22	2016			± 125.0	DON	19.7
	Sugars	19.1	(61–79 weeks)		(12 samples from 6 batches)		OTA	7.6
	Energy	19.8					AFB1	n.d.
8	Protein	44	5 Nov 2016–8	21.9	ZEN	2131.5	ZEN	n.d.
	Fat	16	Mar 2017			± 57.3	DON	51.3
	Sugars	17.5	(80–96 weeks)		(2 samples from 1 batch)		OTA	7.8
	Energy	18.0					AFB1	n.d.

Nutritional information for the fish feed as declared by the manufacturer. Abbreviations: AFB1 – aflatoxin B1, DON – deoxynivalenol, OTA – ochratoxin A, ZEN – zearalenone; S.D. – standard deviation; n.d. – not detected. The limits of detection for ZEN, DON, OTA, and AFB1 were 2.0, 0.1, 0.25, and 0.25 μg·kg^{-1}, respectively. Number of samples collected from batches of prepared feed and analyzed for mycotoxin content are shown in parentheses under the mean content of ZEN and S.D.

$$FCR = \frac{consumed\ feed\ [g]}{body\ mass\ gained\ during\ the\ feeding\ period\ [g]}$$

$$TGC = \frac{final\ body\ mass^{1/3} - initial\ body\ mass^{1/3}\ [g]}{water\ temperature\ during\ the\ feeding\ period\ [°C\ d]} \cdot 1000$$

2.6. Biometric measurements

The total mass and the length of each sampled individual fish were used to determine the condition factor (CF) according to the equation given by Barton et al. [4]:

$$CF = \frac{body\ mass\ [g]}{length\ [mm]^3}\ 10^5$$

The hepatosomatic index (HSI) was calculated as follows:

$$HSI\ [\%\ \text{body mass}] = \frac{liver\ mass\ [g]}{body\ mass\ [g]}\ 100$$

The arithmetic mean, unlike other measures, provides information about the total weight gain of all fish fed with the same feed, which is important information from a practical perspective (e.g. Ref. [59]). Thus, because standard approaches to dealing with skewed distributions like those of the biometric measures in our study (i.e. log-transformations or rank-based approaches like the Mann-Whitney *U* test) may provide misleading conclusions, we chose to bootstrap confidence intervals for the mean differences in selected biometric measures between groups in our study. To perform this analysis, the boot package [6] in R version 3.4.0 [48] was used to bootstrap 95% confidence intervals (95% C.I.) for the mean differences between the exposed and control groups. Bias corrected and accelerated confidence intervals were chosen based on their suitability for this kind of analysis [7], and calculated based on 10000 replicates. If the confidence interval did not include zero, the difference between groups was considered significant. An example of the R script that was used for these calculations is in Supplement 3.

2.7. Hematological and biochemical analyses in blood

Blood was used to prepare blood smears (2 smears per fish). Hematological analysis was performed according to standard methods described in Svobodova et al. [55] in order to determine erythrocyte count, hemoglobin concentration, hematocrit, mean erythrocyte volume, mean corpuscular hemoglobin concentration, mean corpuscular

hemoglobin content, leukocyte count and the differential leukocyte count (leukogram).

Plasma samples were analyzed with a Catalyst Dx Chemistry Analyzer (Idexx Lab; USA) using dedicated test slides (custom panels). The following biochemical measurements were performed: glucose, total protein, albumin, aspartate aminotransferase, alkaline phosphatase, cholesterol, triglycerides, and ammonia. Globulin was calculated by subtracting the albumin from the total protein. Each plasma sample was thawed only once at room temperature and all of the above measurements were performed at once to eliminate multiple freezing/thawing cycles.

The significance of differences in the blood measurements between the two experimental groups of fish (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used. All analyses were performed using SPSS Statistics 24 (IBM), and differences were considered to be significant at $p < 0.05$ (SPSS).

2.8. Histological analysis of tissue sections

The fragments of the tissues were fixed in Bouin's fluid, dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, and then sliced into 4–5 μm sections with a RM 2155 rotational microtome (LEICA Microsystems, Wetzlar, Germany). Cross-sections of tissues were stained with the hematoxyline and eosin.

For additional cytological characterization of the liver, the diameters of 50 hepatocytes and their nuclei from each specimen were measured. The number of hepatocytes was established in a field with a surface area of 2500 μm^2 (50 × 50 μm). Twenty such fields were measured for each individual. In the case of the head kidney and spleen, the number of melanomacrophage centers was counted in 25 fields for each individual. These measurements were performed with a LEICA DM 3000 light microscope and LEICA QWin Pro micro image analysis software (LEICA Microsystems AG, Heerbrugg, Switzerland).

The statistical significance of differences of the diameters between the two experimental groups (control vs. exposed) was assessed using a two-tailed Welch's *t*-test and considered to be significant at $p < 0.05$ (SPSS).

2.9. ELISA for cytokines

Fragments of the liver, head kidney, spleen were preserved in RNA*later* solution (Sigma-Aldrich; Germany) and stored in −20 °C until extraction; 24 individuals were sampled from each group. Then, the same mass of tissue was taken from each sample, and 4 of these portions were combined and homogenized, so that there were 6 measurements for each group.

The preserved tissues were homogenized in ice-cold PBS buffer with 0.5% sodium citrate, 0.05% Tween 20 (Sigma-Aldrich) and protease inhibitor (Roche) using a homogenizer (Omni International). The homogenate was centrifuged at 20,000 g for 1 h (Eppendorf 5804R centrifuge) and the supernatants were stored at −80 °C. Protein concentration was determined using the Bradford method. The contents of cytokines in the supernatants were measured using commercial ELISA kits designed for fish interleukins (Cat. No. IL-4: 201-00-0037, IL-12: 201-00-0020, IL-17: 201-00-0315, and IFN-γ: 201-00-0004; Sunred, China), following the manufacturer's protocol. The absorbance was measured using a Infinite M200 plate spectrophotometer (TECAN; Switzerland) at a wavelength of 450 nm. The content of cytokines was expressed in relation to the total protein content ($pg \cdot mg^{-1}$).

The significance of differences in the content of the cytokines between the two experimental groups of fish (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used; any differences were considered to be significant at $p < 0.05$ (SPSS). As a measure of the size of the effect of ZEN exposure on cytokine expression, Cohen's d was calculated, then transformed to Hedges' g to correct the result for small sample sizes [15].

2.10. PCR detection of Tetracapsuloides bryosalmonae

To test for the presence of *T. bryosalmonae,* RNA*later*-preserved specimens of the macroscopically-changed trunk kidney sections were used to isolate genomic DNA using a Genomic Mini AX Tissue Spin kit, according to the manufacturer's instructions (A&A Biotechnology; Poland). The isolated gDNA samples were used as template in PCR with primers specific to the 18S rDNA sequence of the pathogen (forward: 5′-GGA CAC TGC ATG TGC TGC ATA GT-3′ and reverse: 5′-CCA TGC TAG AAT GTC CAG GCA CT-3′ designed by Ref. [22]. The PCR mixture with each sample contained 10 μL 2X DreamTaq Green PCR Master Mix (ThermoFisher Scientific; USA), 0.25 μM forward and reverse primer, 1 μL (~50 ng) isolated gDNA solution as template, and nuclease-free water for a final volume of 20 μL. The reaction was carried out with the following thermal profile: 95 °C for 2 min, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and final elongation at 72 °C for 5 min. PCR products were electrophoresed in 1.5% agarose gel stained with Midori Green Advanced (Nippon Genetics Europe; Germany), and visualized under UV light. Samples demonstrating a homogenous amplicon at the expected length of 215 bp [22] were scored as positives.

2.11. HPLC analysis of mycotoxins

At 72 weeks of the study, samples were taken from the intestines, liver and muscles of 4 fish from each tank. The samples from each respective organ were then pooled, so that the 4 samples from each tank were combined into one homogenate. Thus, there were 4 pooled homogenates of each organ from each group (i.e. control, exposed).

To estimate the content of free and glucuronidated residuals of ZEN in these homogenates and in the system water, we first separated the residuals from the homogenates with ZearalaTest^{WB} immunoaffinity columns (Vicam; USA). Then, we measured the amount of the residuals with high performance liquid chromatography (HPLC) with fluorescence detection (FLD), according to the protocol described by Woźny et al. [64].

The contents of mycotoxins in the fish feed were estimated using the following procedures:

2.11.1. Deoxinivalenol (DON)

50 g of the fish feed was homogenized with 10 g polyethylene glycol (Mr 8000) in 200 mL distilled water using a CAT X1030D homogenizer at high speed for 3 min. The homogenate was filtered through DONtest fluted filter paper (Vicam), and 6.0 mL of the resulting filtrate was passed through a DONtest-HPLC immunoaffinity column (Vicam) at a flow-rate of 1–2 $drops \cdot s^{-1}$. The column was washed once with 5.0 mL water, and then eluted with 2.0 mL methanol. The methanol eluate was collected in a glass cuvette, evaporated at 50 °C, and the residue was redissolved in 300 μL acetonitrile-water (10:90, v/v) mobile phase. 50 μL of this solution were injected into an into an Agilent 1100 series HPLC system (Supelcosil LC-18, 5 μm, 15 cm x 4.6) with a G1314A UV detector set at 218 nm. The limit of detection (LOD) for DON was 0.10 $\mu g \cdot kg^{-1}$, with a mean recovery of 80%.

2.11.2. Aflatoxin B1 (AFB1) and ochratoxin (OTA)

25g of the fish feed was homogenized with 5 g NaCl for 3 min in a 150 mL methanol:water solution (80:20, v/v), and the homogenate was filtered through fluted filter paper. 10 mL of the filtered extract was diluted with 40 mL of distilled water, mixed well and filtered through microfiber filter paper. Then, 10.0 mL of this extract was passed through an AflaOchra HPLC immunoaffinity column (Vicam). The column was washed once with 1.0 mL water, and then eluted with 2.0 mL methanol. The eluate was evaporated at 50 °C, and then redissolved in 500 μL water:acetonitrile:acetic acid (99:99:2, v/v) mobile

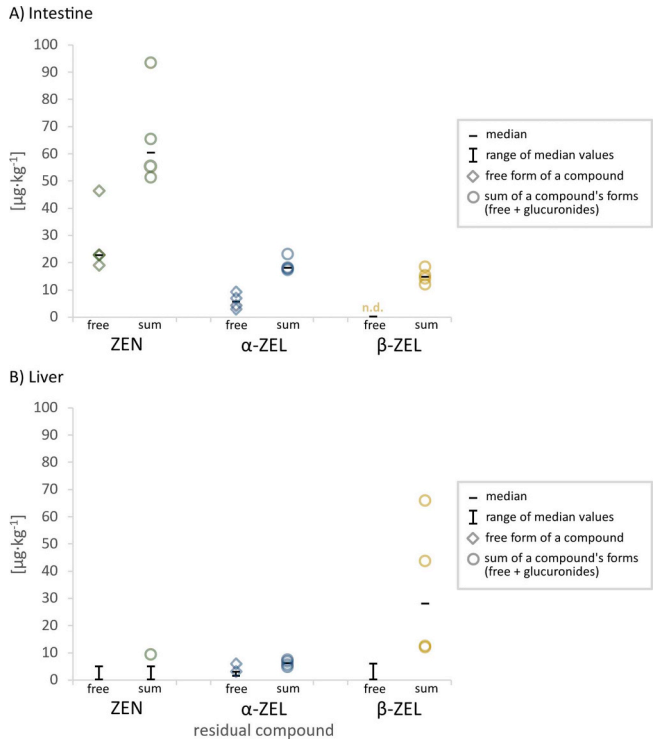

Fig. 2. Content of zearalenone (ZEN; green) and its major metabolites, α- and β-zeranol (α- and β-ZEL; blue and yellow, respectively) in the intestine (A) and the liver (B) of rainbow trout fed for 72 weeks with feed contaminated with ZEN at a dose of 2 mg·kg^{-1} feed (exposed group). The samples were taken from the respective organs of 4 fish from each of the 4 tanks in each group. Each individual point (diamond or circle) represents the value from a pool of samples that were collected from 4 fish from one tank and combined into one homogenate. Thus, there were 4 pooled homogenates of each respective organ from each group (i.e. control or exposed). Horizontal lines show median contents of the compounds; diamonds and circles show individual observations of free forms of compounds, and the sum of free and glucuronidated forms, respectively. Whiskers show the range of possible median values when trace amounts of the compounds were present (below the detection limit of the method). The analysis did not indicate that ZEN or its metabolites were present in the white muscles of the exposed group or in any of the samples collected from the control group (fed with blank feed). Abbreviations: n. d. – not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phase. 50 µL of this solution were injected into an Agilent 1100 series HPLC system (Hypersil ODS column 5 µm, 4.6 mm × 250 mm) with an Agilent G1314A FLD G1321A detector (AFB1 detection wavelength: 360 nm excitation and 440 nm emission; OTA detection wavelength: 333 nm excitation and 477 nm emission). For both AFB1 and OTA, the limit of detection (LOD) was 0.25 µg·kg^{-1}, and the mean recovery was 70%.

2.11.3. Zearalenone (ZEN)

25g of the fish feed was homogenized with 5 g NaCl for 3 min in a 150.0 mL acetonitrile:water solution (90:10, v/v), and the homogenate was filtered through fluted filter paper. 10 mL of the filtered extract was diluted with 40 mL of distilled water, mixed well and filtered through a 0.2 µm disposable filter (Macherey-Nagel, Düren; Germany). Next, 10.0 mL of this extract was passed through a ZearalaTest WB immunoaffinity column (Vicam). The column was washed once with 10.0 mL water, and eluted with 2.0 mL methanol. The eluate was evaporated at 50 °C, and then re-dissolved in 500 µL water:-acetonitrile:methanol (46:46:8, v/v) mobile phase. 20 µL of this solution were injected into the HPLC-FLD system, which was set at an excitation wavelength of 218 nm and an emission wavelength of 438 nm. For ZEN, the limit of detection (LOD) was 2 µg·kg^{-1}, and the mean recovery was 85%.

3. Results

3.1. Content of ZEN and other mycotoxins in fish feed and water

HPLC analysis of the control feed indicated background contamination with ZEN in the first 4 size-fractions of the feed (Table 1). This means that the control fishes consumed a mean ZEN content of 12.3 µg·kg^{-1} total feed (0.6% of the EC guidance value) over the 96 weeks of the experiment. Mean contents of DON and OTA in the control feed were 28.8 and 7.3 µg·kg^{-1} total feed, respectively. AFB1 was not found in any of the analyzed fish feed samples (LOD; < 0.25 µg·kg^{-1}).

When the control feed was enriched with ZEN, the HPLC analysis confirmed that the prepared feed had an overall mean ZEN content of 2040.6 (min. 1876.0 – max. 2131.5) µg·kg^{-1} total feed (Table 1). This is 102% of the EC guidance value for animals other than pigs, cows, sheep, and goats [14]. It should be emphasized that because the ZEN-enriched feed was produced from the same feed that was used for the control fish, background contamination with DON and OTA was the same in both feeds.

Based on the actual content of ZEN in each fraction of the feed (Table 1) and hatchery data on daily feed portions and actual masses of fish in each tank (Supplement 1), we also calculated the dose at which the fish were exposed during the study in terms of mass of ZEN to body mass of the fish (Supplement 4). Although the amount of ZEN fluctuated seasonally, it generally decreased over time, which was expected under the applied feeding regime (Section 2.3). In the exposed group, the daily dose of ZEN ranged from ~60 µg·kg^{-1} body mass in the beginning of the feeding trial to ~5 µg·kg^{-1} body mass in the end (Supplement 4). Although, due to background contamination, ZEN was also present in the control (blank) feed during the first 40 weeks (Table 1), the highest daily doses of ZEN in the control group only reached 3.4 µg·kg^{-1} body mass during the first 4 weeks of the study (Supplement 4).

Water samples collected each month from the hatchery inlets were found to be free of ZEN (LOD; < 2.0 µg·100 dm^{-3}), which excludes the water supply as a source of ZEN contamination.

3.2. ZEN and its metabolites in fish tissues

In order to evaluate whether dietary ZEN was absorbed by the fish, 4 weeks after beginning the feeding trial, we collected eight individual fish fry (~0.8 g each; 6 weeks old) from the control group and eight from the exposed group (2 fish per tank) and measured the content of free ZEN in their whole-body homogenates. The analysis indicated ZEN in all samples collected from the exposed group at a median content of 4.5 µg·kg^{-1} body weight (min – max: 2.6–12.6 µg·kg^{-1}). At the same time, samples collected from the control group were found to be free of ZEN (LOD; < 2.0 µg·kg^{-1}).

To gain insight into the metabolism of ZEN and its distribution in fish tissues, after 72 weeks of the experiment, we also measured the content of free and glucuronidated forms of ZEN and its major metabolites (α- and β-ZEL) in the intestine, the liver, and the white muscles of the exposed fish (Fig. 2). The highest content of ZEN was measured in the intestine of the exposed fish (Fig. 2A), with a median content of 60.5 µg·kg^{-1} tissue, of which 38% (22.8 µg·kg^{-1}) was found to be in the unmetabolized (free) form. Of the two major metabolites of ZEN that were investigated in the intestine, α-ZEL was measured at a median content of 18.1 µg·kg^{-1} tissue, of which 32% (5.7 µg·kg^{-1}) was in the free form. β-ZEL was found only in the glucuronidated form at a median content of 14.8 µg·kg^{-1}.

In contrast to the intestine, only trace amounts of ZEN were found in the liver (Fig. 2B). β-ZEL predominated at a median content of 28.2 µg·kg^{-1} tissue, of which most was present in the glucuronidated

form. Notably, α-ZEL was measured at a median content of 6.2 μg·kg^{-1}, of which only trace levels were found in the free form (median values ranged from 1.6 to 3.1 μg·kg^{-1}).

The HPLC analysis did not indicate the presence of ZEN or its metabolites in the white muscles of the exposed group or in any samples collected from the control group (< LOD; data not shown).

Together, these results show that the fish from the exposed group absorbed the mycotoxin into the digestive system and metabolized it predominantly to the glucuronidated forms. However, neither the parent compound nor the metabolites were transferred to the fishes' muscles.

3.3. Conditions at the hatchery, fish behavior and mortality

The temperature and oxygen concentration of the water supply fluctuated with the seasons (Supplement 1), as expected in a surface-water flow-through hatchery system. Except for a short period of food deprivation during the wintertime when the water temperature dropped below 2.0 °C, all fish were fed on a regular basis. Minor signs of fish stress were observed throughout the study (i.e. crowding in the tanks' corners and occasional reduction of feed consumption), usually related to the reductions of fish stock or transfers of fish from smaller to larger tanks. However, these signs occurred rarely and were observed in both experimental groups (Supplement 1). Throughout the whole feeding trial, the actual mass of fish in each tank did not exceed the maximum recommended stock density [23].

As expected, some fish died in both groups throughout the study. Survival analysis indicated that several tanks differed significantly with regard to time to death, both within and between experimental groups (Supplement 5A). However, although the cumulative survival in the exposed group was lower than that in the control group (88.12 vs. 90.85%), survival time did not differ significantly between these two groups of fish ($p > 0.05$; Supplement 5B).

3.4. Fish growth and feed intake

Collective checkweighting of all fish from each tank at regular intervals showed that the gain in mass of fish in the exposed group was larger than that of fish in the control group (Supplement 1). This gross observation was further confirmed by additional, detailed biometric measurements of individual fish at selected timepoints (Fig. 3). After 60 weeks of exposure to ZEN, the total mass and length of the exposed fish were significantly higher than those of fish in the control group, and these measurements remained significantly higher in the exposed group until the end of the study. For example, after 79 weeks of feeding, an average fish from the exposed group weighed 777.4 g and measured 35.9 cm, which was 110.5 g larger (95% C.I. for the difference, 68.0–150.7 g) and 1.5 cm longer (95% C.I., 1.0–2.1 cm) than an average fish from the control group (Fig. 3). With the exception of condition factor at 96 weeks, all other biometric measures were significantly higher in the exposed group than in the control group (Supplement 6).

Routine monitoring of feed consumption in each tank indicated higher feeding efficiency in the exposed group than in the control group (Supplement 1). To investigate the feeding efficiency and growth rate of the fish, the data acquired at the rearing facility (Supplement 1) was used to calculate FCR and TGC values for the 6 periods that ended with detailed biometric measurements (Fig. 4). For most of the study, the exposed group had a lower FCR and a higher TGC than the control group. However, during weeks 72–96 of the study, the FCR was lower in the control group than in the exposed group (Fig. 4A). In addition, during weeks 79–96, the TGC was higher in the control group (Fig. 4B).

Taken together, these results show that the fish exposed to ZEN had higher growth rates and higher feeding efficiency than the fish in the control group throughout the majority of the experiment. Furthermore, although the FCR and TGC of the control fish were better in the last few weeks of the feeding trial, the exposed fish were still substantially larger.

3.5. Macroscopic, histological, cytological, and molecular observations

3.5.1. Trunk kidney

To investigate whether long-term feed-borne exposure of fish to ZEN affected their health status, selected organs involved in metabolism and the immune response were examined after 72 weeks of the study. Visual inspection of the body cavity showed that most of the examined fish from both groups had typical trunk kidney (mesonephros) structure, but 3 of the 24 fish from the exposed group had distinct macroscopic changes in their trunk kidneys. At this point, the 12 extra fish from each group that had been frozen were investigated and 2 of the ZEN-exposed fish exhibited the macroscopic changes, but none of the control fish. Thus, in total, 5 out of 36 fish in the exposed group showed macroscopic trunk-kidney changes, but none of the fish in the control group.

The macroscopic kidney changes consisted of reddish spots (Fig. 5B), whiteish spots (Fig. 5C), or translucent, whiteish nodules (Fig. 5D) with diameters from 3 to 11 mm. These nodules were different to Stannius bodies, both macroscopically, and microscopically.

To gain insight into the histopathological details of the observed macroscopic changes, we used H&E-stained sections (Fig. 6). Sections collected from parts of the trunk kidney without macroscopic changes showed typical histological structure, containing nephrons with renal corpuscles, distal and proximal convoluted segments, and collecting ducts. These elements were surrounded by interstitial tissue composed of hematopoietic cells and abundant capillaries (Fig. 6A and B). The distal and proximal convoluted tubules were composed of cuboidal epithelial cells with cilia, but in the proximal parts, densely arranged microvilli were visible on the apical surfaces. The inner layer of the collecting ducts and ureters were composed of columnar epithelial cells. Two other components were recognizable in the ureters: connective tissue and circular fibres of smooth muscle (Fig. 6B).

In contrast, tissue sections of the changes that were visible as reddish spots (Fig. 5B) displayed disorganized kidney morphology with numerous inflammatory areas as well as granulomatous structures. Infiltration of the tissue by erythrocytes (and partly by thrombocytes) from ruptured blood vessels was also frequently observed. Excretory tubules had almost disappeared, with only a few rudimentary tubules left. In sections of areas with whiteish nodules (Fig. 5D), excretory tubules were largely absent, but occasionally, regenerating tubules were visible. An infiltration of the tissue by erythrocytes was absent. In some parts of the inflammatory areas, connective tissue, including fibrocytes and intertubular reticular cells, was arranged in circular structures forming granulomatous structures (Fig. 6C and D). On some sections, large melanomacrophage aggregates with hemosiderin were present in the inflammatory regions. There was no clear demarcation between the inflammatory regions and typical kidney tissue regions; instead, they were intermingled without a fibrous sheet or capsule separating them. Overall, the macroscopic changes found in the trunk kidney of these fish indicated massive kidney inflammation/nephritis with granule formation, leading to loss of typical kidney structure and fibrosis.

The histopathological changes resembled an infection with the myxozoan parasite *Tetracapsuloides bryosalmonae*, the causative agent of Proliferative Kidney Disease (PKD). From the H&E sections of the trunk kidney, the parasite could not be unequivocally identified, although some of the granulomatous structures had large cells that resembled the parasite in their centers (Fig. 6D). To exclude fungal or bacterial infection, additional stains with the Grocott and Ziehl-Neelsen methods were performed, which yielded negative results. Therefore, to determine whether *T. bryosalmonae* were present, we extracted genomic DNA from the macroscopically-changed tissue fragments and used the samples in PCR with primers specific to the 18S rDNA of this pathogen. Unfortunately, we could not use PCR to analyze the samples from the asymptomatic fish because we only fixed the samples with macroscopic changes for further analysis. However, the analysis indicated that the

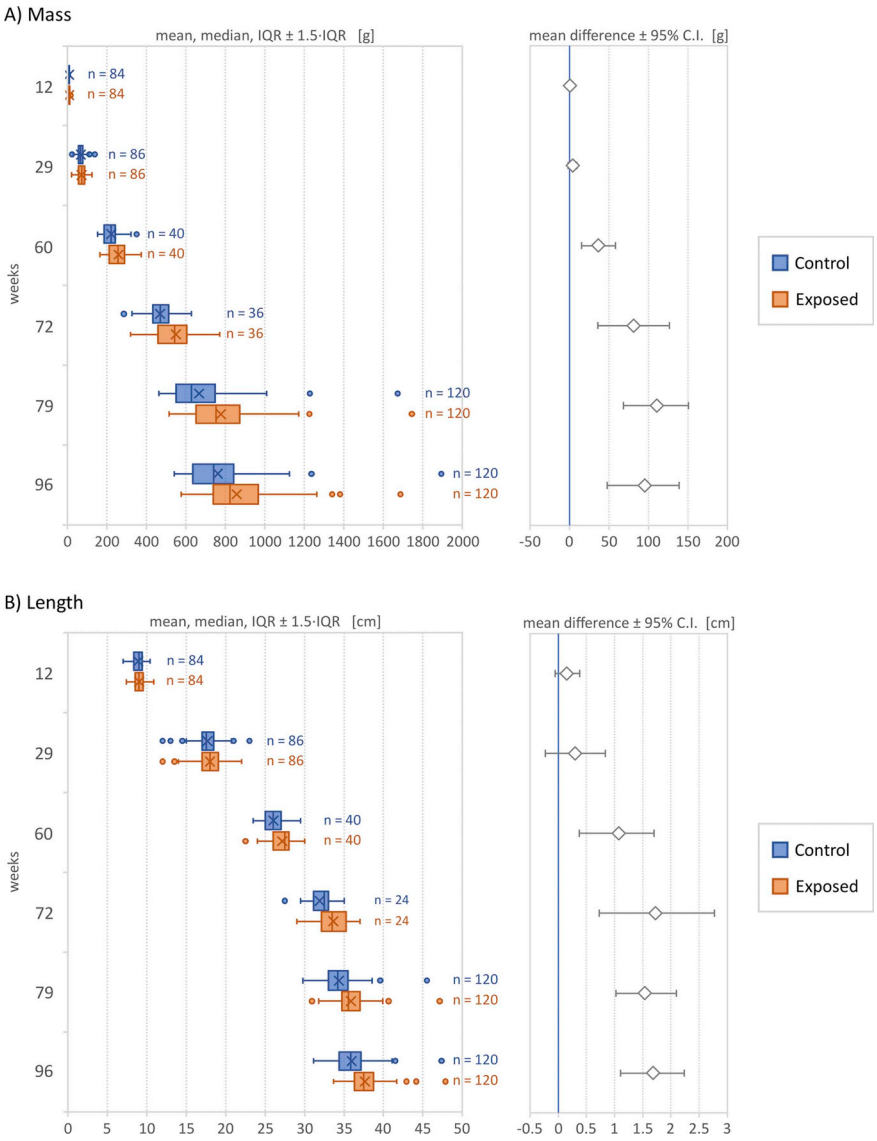

Fig. 3. Total mass (A) and length (B) at 12, 29, 60, 72, 79, and 96 weeks of rainbow trout exposed to zearalenone at a dose of 2 mg·kg^{-1} feed (orange) and of control group (blue). Box plots in the left panel show mean (X), median (vertical line), interquartile range (IQR; box), extent of observations within 1.5·IQR of the box (whiskers), and outliers (dots). Diamonds in the right panel show mean difference between experimental groups ± 95% confidence interval (C.I.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pathogen was indeed present in the macroscopically-changed trunk kidney sections collected from the exposed fish (Fig. 7), strongly suggesting that the kidney inflammation was due to *T. bryosalmonae* infection.

3.5.2. Head kidney

The head kidney is located in an extra-coelomic position in the pharyngeal (cranial) region. This organ contained predominantly hemopoietic and lymphoid cells which were more clearly separated in the fish from the control group than in those from the exposed group (Supplement 7A and B). The hemopoietic tissue had series of blast cells and mature blood cells, such as erythrocytes, as well as large and small lymphocytes. Although identification and classification of blood cells in hemopoietic and lymphoid tissue were hampered by the methods of tissue fixation (Bouin's solution) and section staining (H&E), monocytes (observed as large cells with an indented nucleus and cytoplasm containing numerous vesicles of different sizes) were detected in both experimental groups (Supplement 7C). Additional quantitative analysis of a limited number of fish (n = 7 per group) indicated no significant difference between groups with regard to the number of melano-macrophage centers ($p > 0.05$; Supplement 8).

3.5.3. Spleen

Splenic tissue of fish from both groups was composed of a typical mixture of tissue structures: erythrocyte-rich (red) or lymphocyte-rich (white) pulp, ellipsoids, and melano-macrophages centers. Separation between the hematopoietic red pulp and lymphopoietic white pulp was less distinct in the exposed group than in the control group (Supplement 9A and B). Arterioles that were divided by splenic ellipsoids, forming dense-walled capillaries, were more frequently visible in fish from the exposed group than in fish from the control group (Supplement 9D). Melano-macrophage centers (composed of granules of melanin and phagocytosed material) were typically adjacent to blood vessels and scattered randomly in the splenic tissue of both experimental groups (Supplement 9). Although the difference was not statistically significant, the number of melano-macrophage centers was slightly higher in the exposed group than in the control group ($p = 0.176$; Supplement 8).

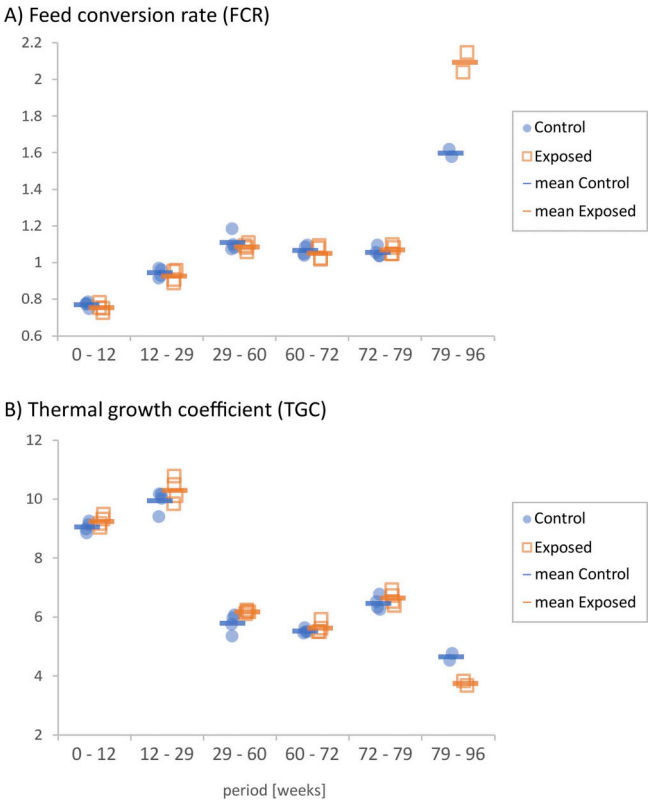

Fig. 4. Feed conversion rate (FCR; A) and thermal growth coefficient (TGC; B) for the group of rainbow trout fed with feed contaminated with zearalenone (ZEN; exposed) at a dose of 2 mg·kg^{-1} feed (orange squares) and for the control group fed with blank feed (blue circles) at selected periods. Point markings (squares or circles) represent FCR and TGC values calculated for individual fish tanks within the given period, whereas horizontal lines indicate means for the groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5.4. Liver

No apparent differences between the appearance of the livers of fish in the exposed group and that of fish in the control group were observed. Also, the hepatosomatic index did not differ significantly between the two groups ($p > 0.05$; Supplement 10).

Histologically, in most of the fish from both groups, the hepatocytes had polygonal shapes and were arranged in rosettes with capillary blood vessels in their central part (Supplement 11A and B). No necrotic areas of liver parenchyma, or inclusions of fibrous connective tissue, or any other major histopathological changes were found in the liver of fish from either group (n = 24 per group). However, hepatocyte vacuolation (conspicuous fat storage) was frequently observed in the liver of fish from both groups, and the vacuolation appeared to be more extensive in the control than in the exposed group (Supplement 11C). Despite the extensive hepatocyte vacuolation in the control group, the observed hepatocyte diameters were virtually identical in both groups ($p = 0.725$; Supplement 10). However, the hepatocytes of all the examined fish from the exposed group had slightly larger nucleus diameters than fish from the control group ($p = 0.013$; Supplement 10). In 4 of 24 (16.7%) histologically-examined fish from the exposed group, heterogenous liver structure with more frequent blood congestions was found. In these 4 fish, some hepatocytes did not have typical polygonal shapes and additional extracellular vacuolization with lipid droplets of various sizes were also observed (Supplement 11D).

3.6. Biochemical indicators in blood plasma

To support the histological analysis, plasma separated from the blood was examined for changes in the levels of selected biomarkers (Table 2). The ratio of albumin/globulin was significantly higher in the plasma of fish from the exposed group than in that of the control group ($p = 0.005$). Although the difference in globulin concentration between the two groups did not reach the threshold of statistical significance ($p = 0.097$), the observed increase in globulin concentration in the exposed group is likely to be the cause of the difference in albumin/globulin ratio between the two groups. There were no other significant

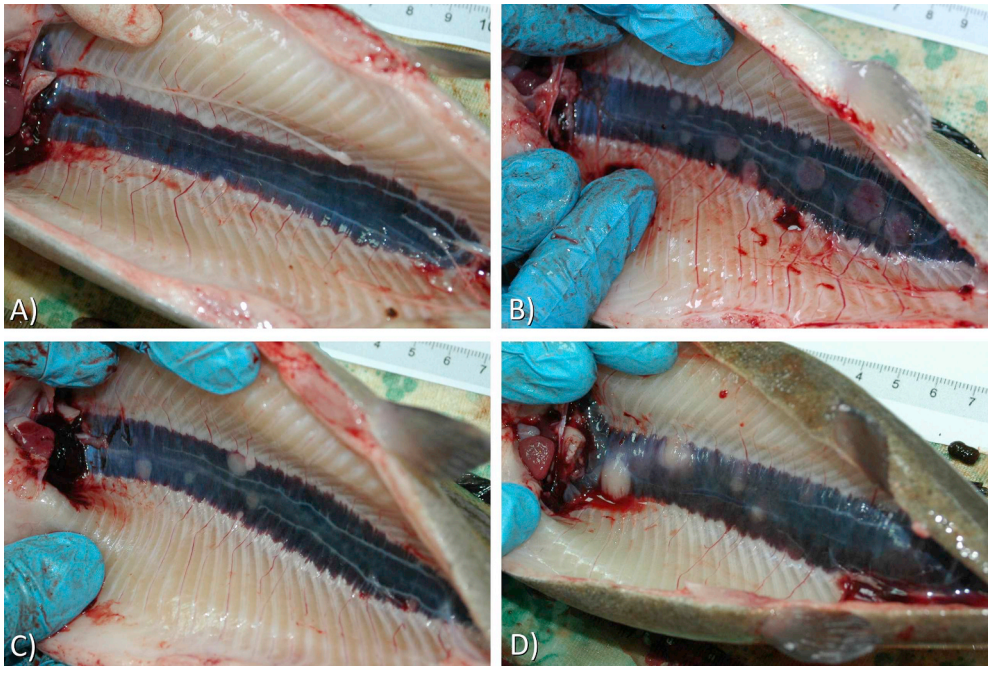

Fig. 5. Macroscopic changes in the trunk kidney of rainbow trout fed with feed contaminated with zearalenone (ZEN; exposed) for 72 weeks at a dose of 2 mg·kg^{-1} feed. The changes were observed as reddish spots (B), whiteish spots (C), or nodules (D) in 5 of 36 fish sampled from the exposed group. None of the 36 fish sampled from the control group, which were fed with blank feed, exhibited these changes in their kidneys (A).

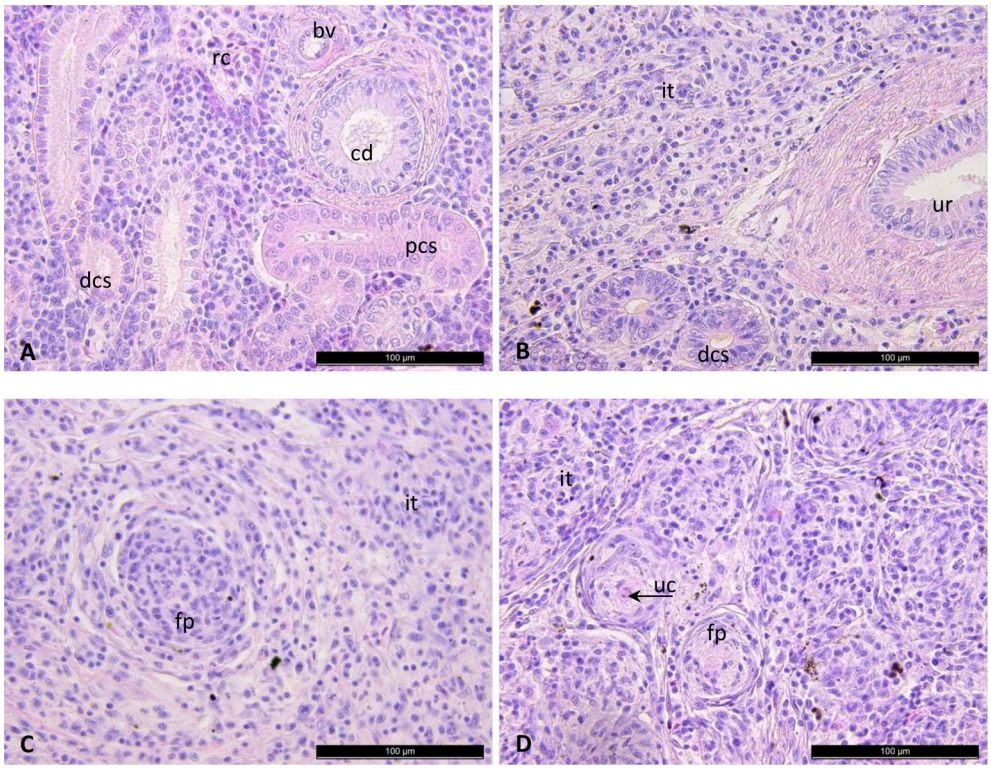

Fig. 6. Histopathological changes in the trunk kidney of rainbow trout exposed to zearalenone for 72 weeks at a dose of 2 mg·kg^{-1} feed. The tissue specimens were collected from trunk kidney sections without any macroscopic changes (A, B), and from sections with reddish or whiteish spots and nodules (C, D; also presented in Fig. 5), and then stained using the H&E method. Abbreviations: bv – blood vessel; cd – collecting duct; dcs – distal convoluted segment; fp – fibrosis of kidney parenchyma; it – interstitial tissue; pcs – proximal convoluted segment; rc – renal corpuscle; uc – unidentified cell (suspected spore of *Tetracapsuloides bryosalmonae*); ur – ureter. Black bar corresponds to 100 µm.

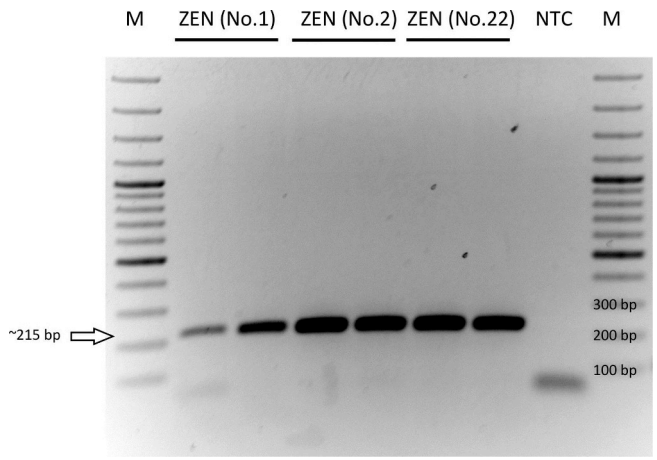

Fig. 7. PCR detection of *Tetracapsuloides bryosalmonae* in macroscopically-changed trunk kidney samples collected from rainbow trout exposed to zearalenone (ZEN). *T. bryosalmonae* 18S rDNA amplicons (at the length of 215 bp; arrow) were obtained from amplification of the genomic DNA extracted from 6 samples (i.e. whiteish or reddish spots or nodules) that were collected from 3 exposed fish (2 samples from each fish) with the macroscopic changes (numbers in parentheses are used to identify each individual fish). PCR products were electrophoresed in 1.5% agarose gel stained with Midori Green Advanced (Nippon Genetics Europe; Germany), and visualized under UV light. Abbreviations: M – molecular weight marker, GeneRuler™ 100 bp Plus DNA Ladder (Thermofisher Scientific); NTC – negative (no template) control.

differences in the examined biochemical indices (Table 2). The lack of distinct changes in the plasma biomarkers of fish from the exposed group confirms that the observed histopathological changes were limited. However, the imbalance in albumin/globulin production in the plasma of fish exposed to ZEN suggests its immunomodulatory effect.

3.7. Hematological indices and blood cell morphology

Selected hematological indices were measured in the blood samples collected after 72 weeks of the study (Table 3). Although the hemoglobin concentration in the blood of fish from the exposed group was significantly higher than that of fish from the exposed group ($p = 0.037$), this difference was small. There were no other significant differences in the examined hematological indices (Table 3).

Additional analysis of the blood smears indicated significant differences in the white blood cells populations between the two groups of fish (Table 4). The amount of B lymphocytes in the blood of fish from the exposed group was only about one-quarter of what it was in the blood of fish from the control group ($p < 0.001$). In addition, although basophiles were rarely observed in the blood of fish from the control group, none of these cells were observed in the exposed group ($p = 0.039$). Furthermore, although this difference was not statistically significant, the number of monocytes in the blood of the exposed fish was only about one-third of that in the blood of the control fish ($p = 0.129$). In contrast, the number of thrombocytes was more than twice as high in the exposed group as in the control group ($p < 0.001$). Similarly, the number of neutrophils was higher in the exposed group than in the control group, although the difference was not significant ($p > 0.05$). These results demonstrate that exposure to ZEN affected key components of the fishes' adaptive and innate immune system.

3.8. Cytokine protein expression

Although many fish cytokines and transcription factors that regulate the development of particulate immune-cell subsets are often discussed in the context of mammalian biology, the cytokines IL-4, IL-12, IL-17, and IFN-γ are generally considered to be major players in the adaptive immunity of fish, covering the spectrum of the Th1, Th2, and Th17 responses [60]. Thus, to gain more information on the adaptive immune response of the fish exposed to ZEN, we examined the content of these cytokines in the head kidney, spleen, and liver of the rainbow trout

Table 2
Biochemical measurements in the blood of rainbow trout exposed to zearalenone (exposed) at a dose of 2 mg·kg^{-1} feed and in the blood of fish fed blank feed (control) for 72 weeks.

Measurement/Indicator	Groups		Significance of difference (p-value)
	Control n = 24	Exposed n = 24	
Glucose [mmol·L^{-1}]	5.50 ± 1.88 (4.71–6.29)	5.25 ± 1.62 (4.56–5.93)	p = 0.564
Total protein [g·L^{-1}]	38.7 ± 8.9 (34.9–42.4)	41.4 ± 5.9 (38.9–43.9)	p = 0.220
Albumin [g·L^{-1}]	15.1 ± 2.5 (14.1–16.2)	15.1 ± 1.9 (14.3–15.9)	p = 1.000
Globulin [g·L^{-1}]	23.5 ± 6.6 (20.7–26.3)	26.3 ± 4.2 (24.5–28.0)	p = 0.097
Albumin/globulin ratio	**0.70 ± 0.26 (0.59–0.81)**	**0.58 ± 0.05 (0.56–0.60)**	**p = 0.005**
Aspartate aminotransferase [U·L^{-1}]	258 ± 136 (201–316)	240 ± 117 (191–289)	p = 0.375
Alkaline phosphatase [U·L^{-1}]	158 ± 63 (131–185)	169 ± 64 (142–196)	p = 0.544
Cholesterol [mmol·L^{-1}]	5.51 ± 1.34 (4.95–6.07)	5.24 ± 0.89 (4.86–5.61)	p = 0.409
Triglycerides [mmol·L^{-1}]	2.67 ± 1.08 (2.21–3.12)	2.86 ± 0.91 (2.46–3.23)	p = 0.538
Ammonia [μmol L^{-1}]	53.3 ± 52.2 (31.2–75.4)	38.3 ± 20.1 (29.8–46.8)	p = 0.550

Mean values (n = 24 in each group) ± standard deviation, and 95% confidence interval of the mean (in parentheses). Differences between the two experimental groups (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used.

Table 3
Hematological measurements from rainbow trout exposed to zearalenone (exposed) at a dose of 2 mg·kg^{-1} feed and from fish fed with blank feed (control) for 72 weeks.

Measurement	Groups		Significance of difference (p-value)
	Control n = 24	Exposed n = 24	
Hematocrit	0.32 ± 0.03 (0.31–0.34)	0.33 ± 0.03 (0.32–0.35)	p = 0.451
Erythrocyte count [T·L^{-1}]	0.96 ± 0.17 (0.89–1.04)	1.04 ± 0.22 (0.95–1.13)	p = 0.496
Hemoglobin concentration [g·L^{-1}]	**58.9 ± 7.8 (55.6–62.2)**	**63.5 ± 7.1 (60.5–66.5)**	**p = 0.037**
Mean corpuscular volume [fL]	347 ± 82 (313–382)	329 ± 67 (301–358)	p = 0.606
Mean corpuscular hemoglobin [g·L^{-1}]	63.2 ± 16.5 (56.3–70.2)	63.1 ± 12.5 (57.8–68.3)	p = 0.726
Mean corpuscular hemoglobin concentration [pg]	0.18 ± 0.02 (0.17–0.19)	0.19 ± 0.03 (0.18–0.20)	p = 0.079

[T·L^{-1}] = 10^{12} cells per litre of whole blood. Mean values (n = 24 in each group) ± standard deviation, and 95% confidence interval of the mean (in parentheses). Differences between the two experimental groups (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used.

after 72 weeks of the study (Fig. 8).

Generally, the expression of cytokines was lower in the head kidney of fish from the exposed group than in those from the control group (Fig. 8A). IL-17 content was slightly lower in the exposed group (about 80% of that in the control group), but this difference was significant ($p = 0.041$). Also in the same group, IFNγ content was about 70% of what it was in the control group ($p = 0.053$). In contrast to the head kidney, the expression of cytokines in the spleen and the liver was generally higher in the exposed group than in the control group, with the differences being more pronounced in the spleen than in the liver (Fig. 8B and C). The content of IL-17 in the spleen of exposed fish was about 270% of that in the control group ($p = 0.002$; Fig. 8B). Although the expression of the other two investigated cytokines (IL-4 and IFNγ) was also increased after exposure to ZEN, the differences between the groups were found to be significant only in the spleen and not in the liver.

In each of the analyzed organs, the size of the effect of ZEN exposure on IL-17 expression was larger than its effects on the other investigated cytokines. For the differences in IL-17 content, the values of Hedges' g ranged from 1.35 in the liver to 3.35 in the spleen, meaning that the difference between the mean IL-17 content in the control group and that in the exposed group ranged from 1.35 to 3.35 standard deviations, which are large to extremely large effects. Differences in IFNγ content in the head kidney and spleen were the second largest effects in these organs, with Hedge's g values of 1.26 and 2.34, respectively. Together, these results indicate that ZEN exposure modulates cytokine levels in different organs.

Table 4
Leukogram results from rainbow trout exposed to zearalenone (exposed) at a dose of 2 mg·kg^{-1} feed and from fish fed with blank feed (control) for 72 weeks.

Measurement	Groups		Significance of difference (p-value)
	Control n = 24	Exposed n = 24	
White blood cells [G·L^{-1}]	4.25 ± 1.50 (3.62–4.88)	4.33 ± 1.72 (3.61–5.06)	p = 0.859
Total Lymphocytes [G·L^{-1}]	3.88 ± 1.43 (3.27–4.48)	3.89 ± 1.59 (3.22–4.56)	p = 0.981
Small lymphocytes [G·L^{-1}]	3.62 ± 1.41 (3.02–4.21)	3.82 ± 1.55 (3.16–4.47)	p = 0.642
B Lymphocytes [G·L^{-1}]	**0.26 ± 0.17 (0.19–0.33)**	**0.07 ± 0.08 (0.04–0.11)**	**p = 1.2E-5**
Total Neutrophils [G·L^{-1}]	0.36 ± 0.21 (0.27–0.45)	0.44 ± 0.26 (0.33–0.55)	p = 0.257
N Neutrophils [G·L^{-1}]	0.19 ± 0.13 (0.14–0.25)	0.21 ± 0.14 (0.15–0.26)	p = 0.902
S Neutrophils [G·L^{-1}]	0.16 ± 0.13 (0.11–0.22)	0.24 ± 0.18 (0.16–0.31)	p = 0.161
Monocytes [G·L^{-1}]	0.008 ± 0.012 (0.005–0.013)	0.003 ± 0.007 (0.000–0.006)	p = 0.129
Basophiles [G·L^{-1}]	**0.003 ± 0.008 (0.000–0.007)**	**0.000**	**p = 0.039**
Eosinophiles [G·L^{-1}]	0.000	0.000	p = 1.000
Thrombocytes [G·L^{-1}]	**0.52 ± 0.40 (0.36–0.69)**	**1.14 ± 0.54 (0.91–1.37)**	**p = 1.1E-4**

[G·L^{-1}] = 10^{9} cells per litre of whole blood. Mean values (n = 24 in each group) ± standard deviation, and 95% confidence interval of the mean (in parentheses). Differences between the two experimental groups (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used.

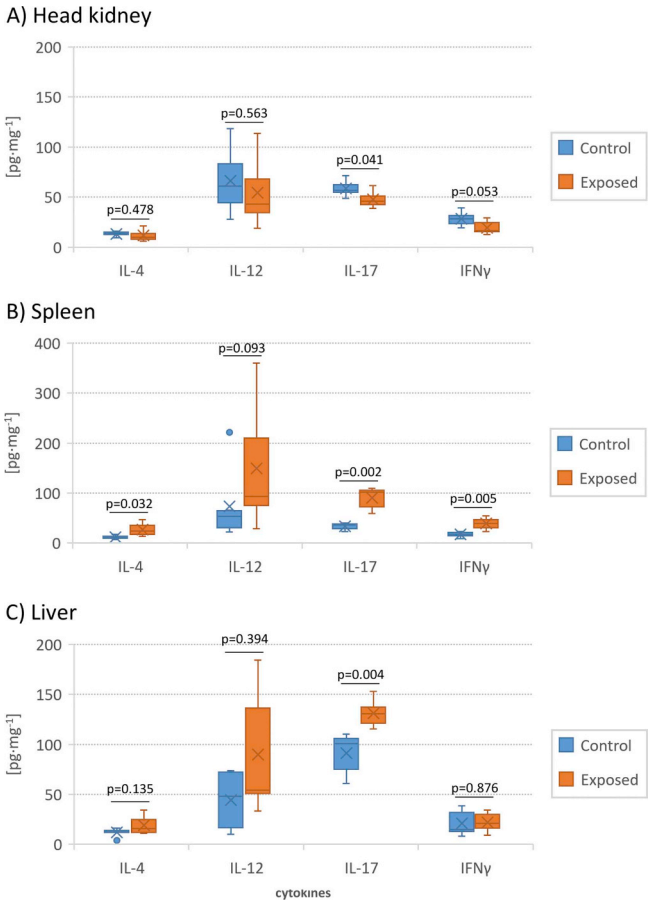

Fig. 8. Content of interleukin 4 (IL-4), 12 (IL-12), and 17 (IL-17), and of interferon γ (IFNγ) in the head kidney (A), spleen (B), and liver (C) of rainbow trout fed for 72 weeks with feed contaminated with zearalenone (exposed; orange) at a dose of 2 mg·kg^{-1} feed or with blank feed (control; blue). The tissues were collected from 24 individuals from each group, but the analysis was performed on pooled samples. Briefly, the same mass of tissue was taken from each sample, and 4 of these portions were combined and homogenized, so that, in total, there were measurements of 6 pooled samples for each group. The content of cytokines was shown in relation to the total protein content (pg·mg^{-1}). Box plots show mean (X), median (horizontal line), interquartile range (IQR; box), extent of observations within 1.5·IQR of the box (whiskers), and outliers (dots). The significance of differences in cytokine contents between the two experimental groups of fish (control vs. exposed) were assessed using a two-tailed Welch's *t*-test, or in the case of non-normally distributed data (Shapiro-Wilk test; $p < 0.05$), a Mann-Whitney *U* test was used. Note the different scales on the vertical axes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. ZEN is absorbed and metabolized in the digestive system but its residues do not accumulate in the muscles of the exposed fish

In order to obtain information on the intake of ZEN from the contaminated feed, we analyzed the tissues of the exposed fish for residual content of ZEN and its major metabolites (α- and β-ZEL) in free (unconjugated) or glucuronidated forms. ZEN was present in the whole-body homogenates of fish fry 4 weeks after the beginning of the experiment (Results section 3.2), as well as in the digestive system of fish after 72 weeks (Fig. 2). Importantly, these compounds were not found in any of the samples collected from the control group of fish fed with blank feed. Therefore, our results show that the presence of ZEN in the tissues of the exposed fish was a result of the feed contamination and that the fish were continuously exposed throughout the study.

Our results are in agreement with previous research on the toxicokinetics of ZEN in orally-exposed rainbow trout, showing that the mycotoxin is fairly rapidly absorbed and primarily metabolized in the fish intestine and liver [31,64]. In addition, our results suggest that water temperature plays a role in the toxicokinetics and metabolic profile of ZEN in fish. This is because, despite the fact that the fish in this study were fed at a slightly higher dose (i.e. 2.0 vs. 1.8 mg·kg^{-1} feed) and for a longer time (72 vs. 10 weeks) than in our previous study [65], the median contents of free forms ZEN and α-ZEL found in the fish intestines in the present study were lower (Fig. 2A) than in our previous study (i.e. 74.5 and 11.1 μg·kg^{-1} tissue, respectively). Furthermore, during the 10 weeks before intestine samples were collected, the fish in the present study were kept in water temperatures almost three times higher than those in our previous study (17.2 vs. 6.5 °C). The metabolic rate of teleost fish increases as temperature increases [11]. Thus, it is likely that the higher temperatures in the present study increased ZEN elimination rate, which resulted in lower content of ZEN and its metabolites in the exposed fish. This may have toxicological implications for aquaculture because the same level of contamination in feed may have different effects, depending on fish species and their optimal water temperature, e.g. in polar and tropical fish species.

The extent to which ZEN and its reductive metabolites (α- and β-ZEL) are conjugated with glucuronic acid (known as glucuronidation) differs between livestock species. For example, laying hens conjugate ZEN and its reductive metabolites to a lesser extent than cattle and pigs, in which less content of these free compounds are found [13]. In the present study, glucuronidated forms of ZEN and its reductive metabolites were more abundant than their unconjugated forms in the intestine and the liver of the fish fed with the contaminated feed (Fig. 2). This observation is in agreement with our previous study on mature rainbow trout which showed that, 96 h after receiving an oral bolus with ZEN, glucuronides constituted over 80% of all residuals found in the liver of the exposed fish [64]. Therefore, the present results confirm that glucuronidation is most likely a major pathway of ZEN metabolism in rainbow trout. Whether the predominance of the glucuronidated metabolites of ZEN affects the sensitivity of rainbow trout to ZEN, and the details of how these metabolites affect the fishes' immune system, should be further investigated to better understand the biological consequences of piscine metabolism of this mycotoxin.

The present finding increases our confidence that exposure of rainbow trout to ZEN at the current EC guidance value does not pose a risk to the consumers of the fish meat. Previously, we had found that ZEN was not present in trout muscles after 10 weeks of exposure at a dose of 90% of the EC guidance value [65]. In the present study, the fish were exposed to ZEN at ~100% of the EC guidance value from the beginning of external feeding until most of them were at market weight (72 weeks of feeding), and kept in typical hatchery conditions for this time. The repetition of our previous finding of no transfer of ZEN to trout muscles even at the higher dose and after the increased length of exposure leads us to conclude that it is highly unlikely that meat from trout exposed to ZEN at the current EC guidance value (i.e. 2 mg·kg^{-1} feed) would be a meaningful source of dietary intake of ZEN, and thus pose a health risk to consumers.

4.2. Feed contaminated with ZEN promoted the growth of the fish

In this study, we found that feed-borne exposure of rainbow trout to ZEN at a dose of 2 mg·kg^{-1} feed increased growth rate and feeding efficiency throughout most of the feeding trial, which at the end of the study, resulted in significantly higher body mass and length of the exposed fish (Figs. 3 and 4; Supplement 6). Although the sensitivity of the HPLC-FLD method only allowed us to determine the content of ZEN, and α- and β-ZEL, and not that of other metabolites of ZEN (i.e. α- and β-ZAL), it has been shown in other species that these metabolites of ZEN are present, and that they have anabolic properties [13]. For this

reason, α-ZAL (α-zearalanol) has been long and widely used in the USA for cattle [50], and it has also been proposed that it be included in fish feed as a growth promoting agent [27]. In a study on rainbow trout, the final weight of fish fed for 21 days with a diet containing α-ZAL at 10 and 20 $mg \cdot kg^{-1}$ for 21 days was 25 and 30% higher, respectively, than that of fish fed with a control diet [29]. α-ZAL has a stronger growth promoting effect than β-ZAL, and it does not appear to promote growth via local action in the tissues because it does not transfer to muscle tissue to a great extent [28]. Instead, it has been shown to promote bone and muscle growth by stimulating the pituitary gland to produce somatotropin, an endogenous growth hormone [58,63]. Thus, we believe that the growth-promoting effects of ZEN that were observed in our study resulted from the anabolic properties of its metabolite, the α-ZAL.

Two other studies with fish have previously investigated the effect of dietary ZEN on the growth performance of fish. These studies were done with premarket-size (~250 g) rainbow trout exposed to ZEN at a dose of 1.81 $mg \cdot kg^{-1}$ feed for 10 weeks [65], and fingerlings (~30 g) of carp exposed to ZEN at doses ranging from 0.33 to 0.80 $mg \cdot kg^{-1}$ feed for 4 weeks [43]. Although Woźny et al. [65] found that the FCR of ZEN-exposed fish was 85% of that of control fish, both of these previous studies found no significant differences in the final weight of fish between the exposed and the control groups at the end of both feeding trials, which might seem to contradict our present finding that ZEN promoted fish growth. However, there are important differences between the present study and these two previous studies that should be considered. First, the fish in the earlier studies were at later developmental stages (i.e. phases of slower growth) and they were exposed to lower doses of ZEN for shorter periods of time than in the present study. Thus, both the lower dose and the shorter length of exposure likely resulted in a less pronounced effect of ZEN exposure. Second, the sample sizes used in the previous studies to estimate the effect were much smaller than that in the present study, which may have caused the previous studies to lack the statistical power to detect a small effect resulting from ZEN exposure at smaller dose and for a shorter period. Thus, we believe that, in light of these important experimental differences, the previous results do not contradict those of the present study, and that the present results suggest that it may be worthwhile to use larger sample sizes to investigate the effects of realistic doses of ZEN and other mycotoxins, i.e. doses that fish are likely to be exposed to under typical feeding conditions.

4.3. Exposure to ZEN affected the immune system of the fish

Sex steroids regulate the immune system of fish. Unlike androgens, which are thought to generally have immunosuppressive effects, the immunomodulatory action of estrogens is rather complex. This complexity is due to numerous factors, including the diversity of estrogen signaling, cell-type–specific microenvironments, hormone concentrations, and physiological status [54]. In general, however, estrogens at low concentrations are believed to stimulate the immune response, whereas at high concentrations, they tend to suppress this response [9,35,56]. For example, recent studies strongly suggest that increased concentrations of sex steroids make fish more vulnerable to infections during their spawning season [56]. Likewise, it has also been shown that endocrine disrupting compounds target the immune system, increasing the susceptibility of fish to disease by interfering with immune system homeostasis [35].

In our study, exposure of rainbow trout to ZEN resulted in marked alterations of their blood cell populations: whereas the amount of B lymphocytes in the blood of the exposed fish was only one-quarter of that in the blood of the untreated fish, the number of thrombocytes was more than twice as high in the exposed group as in the control (Table 4). Moreover, the changes in the leukograms of the exposed fish were accompanied by an altered albumin/globulin ratio in their blood (Table 2). These results indicate that the feed-borne exposure to ZEN affected key components of the fishes' adaptive and innate immune systems, which is likely due to the hormone-mimicking properties of ZEN (or its metabolites). Furthermore, because B lymphocytes and thrombocytes play important roles in defending fish against pathogens [39,41], these findings suggest that the exposure to ZEN may have perturbed the health of the fish.

The alterations in expression of cytokine proteins (IL-4, IL-12, IL-17, and IFNγ) in the immune-related organs of the fish after 72 weeks of exposure to ZEN (Fig. 8) provide further evidence that this mycotoxin has immunomodulatory properties. These alterations were most distinct in the spleen, where expression of IL-4, IL-17, and IFNy was significantly higher in the exposed fish than in the control fish (Fig. 8B). In the liver, although the mean differences between groups were somewhat smaller and only the difference in IL-17 expression was significant, the overall pattern was the same: the observed expression of all four cytokines was higher in the exposed group than in the control group (Fig. 8C). In the head kidney, in contrast, the pattern was reversed: although only the difference in IL-17 expression reached the threshold of statistical significance, the expression of all four cytokines was lower in the exposed group than in the control group (Fig. 8A). These results show that exposure to ZEN has distinct tissue-specific effects on the cell-mediated immunity of the exposed fish, which is in agreement with previous *in vivo* and *in vitro* studies that have found that ZEN has divergent effects on the immune system, acting as an inductor or suppressor of the pro-inflammatory response in different organs or cell cultures (e.g. Refs. [32,47,62]). For example, 18 days of feed-borne exposure of pigs to ZEN (at a dose of 0.316 $mg \cdot kg^{-1}$ feed) induced expression of pro-inflammatory cytokines in the spleen, but also decreased expression of the cytokines in the liver [32,47]. Whether the tissue-specific effects observed here and in other studies are due to altered numbers of particular immune-cell subsets (e.g. Th cells) or changes in their activity remains to be further investigated. Nevertheless, our results suggest that exposure to ZEN may have detrimental effects on immune cell maturation and function.

Among the cytokines investigated in our study, not only was the difference in IL-17 expression statistically significant in all three of the organs that were investigated (Fig. 8), but the size of the effect of ZEN exposure on IL-17 expression was also larger than the mycotoxin's effects on the other investigated cytokines. In mammals, the IL-17 family members (homologues ranging from IL-17A to IL-17F) are considered to play a role in coordinating innate and adaptive immunity. In fish, several homologues of these genes have been found [60,61]. Although little is currently known about the function of these homologues, induced expression of *IL-17A/F2* has been found in the head kidney of rainbow trout infected with viral (VHSV) and bacterial (*Yersinia ruckeri*) pathogens, as well as with the parasite *Tetracapsuloides bryosalmonae* [37]. Furthermore, recombinant IL-17A/F2 protein was found to induce expression of an antimicrobial peptide and pro-inflammatory cytokines in trout spleenocyte culture [37], strongly suggesting that, in fish, this cytokine plays a role in host-defense against pathogen infections [60].

Our results add to available knowledge on the immunomodulatory activity of ZEN in fish species. Whereas in mammals, numerous *in vivo* studies have demonstrated that ZEN affects a wide range of components of both the humoral and the cellular immune responses (e.g. Refs. [5,10,26,32,47]), only a few studies have investigated similar issues in fish. In an experiment with juvenile carp, fish exposed to ZEN at doses of 0.62 and 0.80 $mg \cdot kg^{-1}$ feed for 4 weeks (but not those exposed at 0.33 $mg \cdot kg^{-1}$) had more granulocytes and fewer monocytes in their blood than control fish [43]. Further analysis of leukocytes isolated from the head and the trunk kidney of these fish revealed that exposure to the low and the medium dose of ZEN (i.e. 0.33 and 0.62 $mg \cdot kg^{-1}$) increased the respiratory burst of the immune cells, whereas the higher dose (0.80 $mg \cdot kg^{-1}$) decreased this response [44]. These studies have shown that by stimulating the immune response at low concentrations and suppressing it at high concentration, ZEN may interfere with the immune homeostasis of fish in a manner similar to that of estradiol [54,56].

The immunomodulatory effects of ZEN may have compromised the immune system of the exposed fish, making them more susceptible to *T. bryosalmonae* infection, which in turn, may have caused the histopathological changes observed in the trunk kidneys (Figs. 5 and 6). *T. bryosalmonae* is a myxozoan parasite of salmonids that causes Proliferative Kidney Disease (PKD), which can be a serious problem in fish farms and hatcheries due to increased mortality [25,40]. The disease is characterized by massive inflammation, mainly in the kidney, and provokes a chronic immunological response that often manifests as formation of granulomatous lesions, renal atrophy, or modulation of cytokine synthesis [3,22,40]. Although the disease has a high infection rate [25,40], so that most or all of the fish in the control and exposed groups were probably infected by the parasite, macroscopic changes were only found in the fish sampled from the exposed group, suggesting a link between loss of immune function due to ZEN exposure and development of PKD symptoms. Indeed, it has been found that the course of *T. bryosalmonae* infection is shaped by the immune status of the host fish [3,22], which in turn, is affected by external and internal factors, i.e. water temperature or hormones [56]. For example, even a slight increase in water temperature, from 12 to 15 °C, caused rainbow trout to become immunodeficient, and after exposure to *T. bryosalmonae*, to suffer from a higher prevalence of infection and a higher pathogen burden than fish kept at 12 °C [3]. This study and others [43,44] have shown that feed-borne exposure to ZEN modulates the immune system in fish, suggesting that ZEN (or its metabolites) may interfere with their immune homeostasis. Thus, we believe that the long-term exposure to ZEN may have compromised the immune function of the fish in our study, making them more susceptible to developing symptoms of *T. bryosalmonae* infection, such as the observed massive kidney inflammation. Further research will be necessary to confirm the link between ZEN exposure and the development of clinically relevant symptoms (i.e. pathogen-challenge tests on dietary-exposed fish). However, if ZEN indeed increases the susceptibility of fish to pathogens, this raises the possibility that exposure to ZEN at the existing EC guidance value may have economic repercussions for aquaculture due to treatment costs and increased mortality resulting from diseases like *T. bryosalmonae* infection.

5. Conclusions

Our study demonstrates that the contamination of fish feed with the mycotoxin zearalenone (ZEN) at the European Commission (EC) guidance value (2 $mg \cdot kg^{-1}$ feed) may have implications for sustainable aquaculture. In this study, we show that feed-borne exposure of rainbow trout to ZEN at a dose of 2 $mg \cdot kg^{-1}$ feed resulted in higher feeding efficiency and growth rate, most probably due to the anabolic properties of the ZEN metabolite. Importantly for the consumers of such fish, despite absorption and metabolism of ZEN in the digestive system of the fish that had been exposed for 72 weeks, the mycotoxin's residuals were not transferred to the fishes' muscles. This rules out a potential risk to human health related to the consumption of fish meat after this fish-feeding regimen. However, the increased growth of fish fed with the contaminated feed may come at some cost, as long-term exposure to ZEN may perturb the immune system of the fish and increase their susceptibility to pathogens. Although additional research is necessary to confirm the clinical relevance of the observed immunomodulatory effect of ZEN (i.e. pathogen-challenge tests on dietary-exposed fish), our results raise concerns about the safety of the current recommended EC guidance values for fish and suggest a need to consider lowering these maximum allowable levels of ZEN in feed and feedstuff used in aquaculture.

Conflicts of interest

The authors declare to have no conflict of interest.

Contributors

MW prepared the project's concept; **RR** performed the hatchery operations; **MW, MF, PB** collected the samples; **MW** prepared the contaminated feed and **KO** performed the analysis of residuals; **PH, HS** performed the histological analysis; **PG** performed the biochemical and hematological measurements; **MW** performed the molecular diagnostics of the pathogen; **PW, KO** performed the cytokine expression analysis; **MW, PB, PG, PH, HS** worked on the results interpretation; **MW** wrote the manuscript. All authors have approved the final version of article before its submission.

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Appendix A. Supplementary data

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References

[1] A. Anater, L. Manyes, G. Meca, E. Ferrer, F.B. Luciano, C.T. Pimpão, G. Font, Mycotoxins and their consequences in aquaculture: a review, Aquaculture 451 (2016) 1–10 https://doi.org/10.1016/j.aquaculture.2015.08.022.
[2] A. Arukwe, T. Grotmol, T.B. Haugen, F.R. Knudsen, A. Goksøyr, Fish model for assessing the in vivo estrogenic potency of the mycotoxin zearalenone and its metabolites, Sci. Total Environ. 236 (1999) 1–3 http://doi.org/10.1016/S0048-9697(99)00275-2.
[3] C. Bailey, H. Segner, A. Casanova-Nakayama, T. Wahli, Who needs the hotspot? The effect of temperature on the fish host immune response to Tetracapsuloides bryosalmonae the causative agent of proliferative kidney disease, Fish Shellfish Immunol. 63 (2017) 424–437 https://doi.org/10.1016/j.fsi.2017.02.039.
[4] B.A. Barton, J.D. Morgan, M.M. Vijayan, Physiological and condition related indicators of environmental stress in fish, in: S.M. Adams (Ed.), Biological Indicators of Aquatic Ecosystem Stress, American Society of Fisheries, Bethesda, Maryland, 2002, pp. 111–148.
[5] S.P. Boeira, C.B. Filho, L. Del'Fabbro, S.S. Roman, L.F.F. Royes, M.R. Fighera, C.R. Jessé, M.S. Oliveira, A.F. Furian, Lycopene treatment prevents hematological, reproductive and histopathological damage induced by acute zearalenone administration in male Swiss mice, Exp. Toxicol. Pathol. 66 (2014) 179–185 https://doi.org/10.1016/j.etp.2014.01.002.
[6] A. Canty, B. Ripley, Boot: Bootstrap R (S-plus) Functions. R Package Version 1, (2017), pp. 3–19.
[7] J. Carpenter, J. Bithell, Bootstrap confidence intervals: when, which, what? A practical guide for medical statisticians, Stat. Med. 19 (2000) 1141–1164 https://doi.org/10.1002/(SICI)1097-0258(20000515)19:9%3C1141::AID-SIM479%3E3.0.CO;2-F.
[8] T. Celius, T.B. Haugen, T. Grotmol, B.T. Walther, A sensitive zonagenetic assay for rapid in vitro assessment of estrogenic potency of xenobiotics and mycotoxins, Environ. Health Perspect. 107 (1999) 63–68.
[9] E. Chaves-Pozo, A. García-Ayala, I. Cabas, Effects of sex steroids on fish leukocytes, Biology 7 (2018) 9 https://doi.org/10.3390/biology7010009.
[10] P. Chen, T. Liu, S. Jiang, Z. Yang, L. Huang, F. Liu, Effects of purified zearalenone on selected immunological and histopathologic measurements of spleen in post-weanling gilts, Animal Nutrition 3 (2017) 212–218 https://doi.org/10.1016/j.aninu.2017.04.008.
[11] A. Clarke, N.M. Johnston, Scaling of metabolic rate with body mass and temperature in teleost fish, J. Anim. Ecol. 68 (1999) 893–905 http://doi.org/10.1046/j.1365-2656.1999.00337.x.
[12] CONTAM (European Food Safety Authority; Panel on Contaminants in the Food Chain), Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to zearalenone as undesirable substance in animal feed, EFSA Journal 89 (2004) 1–35.
[13] S. Dänicke, J. Winkler, Invited review: diagnosis of zearalenone (ZEN) exposure of farm animals and transfer of its residues into edible tissues (carry over), Food Chem. Toxicol. 84 (2015) 225–249 https://doi.org/10.1016/j.fct.2015.08.009.
[14] EC (European Commission), Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding, Official Journal of the European Union 23 (8) (2006) 2006 L 229/7–9.
[15] P.D. Ellis, The Essential Guide to Effect Sizes. Statistical Power, Meta-analysis, and the Interpretation of Research Results, Cambridge University Press, New York, 2010, pp. 10–15.

[16] M. Eskola, A. Altieri, J. Galobart, Overview of the activities of the european food safety authority on mycotoxins in food and feed, World Mycotoxin J. 11 (2018) 277–289 https://doi.org/10.3920/WMJ2017.2270.
[17] J. Fink-Gremmels, H. Malekinejad, Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone, Anim. Feed Sci. Technol. 137 (2007) 326–341 http://doi.org/10.1016/j.anifeedsci.2007.06.008.
[18] J. From, G. Rasmussen, A growth model, gastric evaluation and body composition in rainbow trout, Salmo gairdneri Richardson, 1836, Dana 3 (1984) 61–139.
[19] R.A. Gonçalves, U. Hofstetter, D. Schatzmayr, T. Jenkins, Mycotoxins in Southeast Asian aquaculture: plant-based meals and finished feeds, World Mycotoxin J. 11 (2018) 265–275 https://doi.org/10.3920/WMJ2017.2239.
[20] R.A. Gonçalves, K. Naehrer, G.A. Santos, Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: a real risk to aquaculture? Reviews in Aquaculture, https://doi.org/10.1111/raq.12159, (2016).
[21] R.A. Gonçalves, D. Schatzmayr, U. Hofstetter, G.A. Santos, Occurrence of mycotoxins in aquaculture: preliminary overview of Asian and European plant ingredients and finished feeds, World Mycotoxin J. 10 (2017) 183–194 https://doi.org/10.3920/WMJ2016.2111.
[22] B. Gorgoglione, T. Wang, C.J. Secombes, J.W. Holland, Immune gene expression profiling of proliferative kidney disease in rainbow trout Oncorhynchus mykiss reveals a dominance of anti-inflammatory, antibody and T helper cell-like activities, Vet. Res. (Paris) 44 (2013) 55 https://doi.org/10.1186/1297-9716-44-55.
[23] K. Goryczko, Pstrągi Chów I Hodowla Poradnik Hodowcy, Wydawnictwo IRS, Olsztyn, 978-83-60111-23-9, 2008, p. 24.
[24] K. Gromadzka, A. Waśkiewicz, J. Świetlik, J. Bocianowski, P. Goliński, Possible way of zearalenone migration in the agricultural environment, Plant Soil Environ. 61 (2016) 358–363 https://doi.org/10.17221/115/2015-PSE.
[25] R.P. Hedrick, E. MacConnell, P. de Kinkelin, Proliferative kidney disease of salmonid fish, Annu. Rev. Fish Dis. 3 (1993) 277–290 https://doi.org/10.1016/0959-8030(93)90039-E.
[26] I.M. Hueza, P.C.F. Raspantini, L.E.R. Raspantini, A.O. Latorre, S.L. Górniak, Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound, Toxins 6 (2014) 1080–1095 https://doi.org/10.3390/toxins6031080.
[27] M.J. Jacobs, F. Hart, Growth promoting composition for fish and method of using the same, Biotechnol. Adv. 15 (3–4) (1997) 778 https://doi.org/10.1016/S0734-9750(97)88715-0.
[28] S.-H. Jeong, D. Kang, M.-W. Lim, C.S. Kang, H.J. Sung, Risk assessment of growth hormones and antimicrobial residues in meat, Toxicol Res 26 (2010) 301–313 https://doi.org/10.5487/TR.2010.26.4.301.
[29] O. Keleş, A. Candan, T. Bakirel, S.K. Düğenci, The investigation of the anabolic efficiancy and effect on the nonspecific immune system of zeranol in rainbow trout (Oncorhynchus mykiss, Walbaum), Turk. J. Vet. Anim. Sci. 26 (2002) 925–931.
[30] T. Kuiper-Goodman, P.M. Scott, H. Watanabe, Risk assessment of the mycotoxin zearalenone, Regul. Toxicol. Pharmacol. 7 (1987) 253–306 http://doi.org/10.1016/0273-2300(87)90037-7.
[31] A. Laganà, A. Faberi, G. Fago, A. Marino, E. Pastorini, R. Samperi, Application of an innovative matrix solid-phase dispersion-solid-phase extraction-liquid chromatography-tandem mass spectrometry analytical methodology to the study of the metabolism of the estrogenic mycotoxin zearalenone in rainbow trout liver and muscular tissue, Int. J. Environ. Anal. Chem. 84 (2004) 1009–1016 http://doi.org/10.1080/03067310410001730646.
[32] D.E. Marin, G.C. Pistol, I.V. Neagoe, L. Calin, I. Taranu, Effects of zearalenone on oxidative stress and inflammation in weanling piglets, Food Chem. Toxicol. 58 (2013) 408–415 https://doi.org/10.1016/j.fct.2013.05.033.
[33] I. Matejova, Z. Svobodova, J. Vakula, J. Mares, H. Modra, Impact of mycotoxins on aquaculture fish species: a review, J. World Aquacult. Soc. 48 (2017) 186–200 https://doi.org/10.1111/jwas.12371.
[34] M. Metzler, E. Pfeiffer, A.A. Hildebrand, Zearalenone and its metabolites as endocrine disrupting chemicals, World Mycotoxin J. 3 (2010) 385–401 http://doi.org/10.3920/WMJ2010.1244.
[35] S. Milla, S. Depiereux, P. Kestemont, The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review, Ecotoxicology 20 (2011) 305–319 https://doi.org/10.1007/s10646-010-0588-7.
[36] F. Minervini, M.E.D. Aquila, Zearalenone and reproductive function in farm animals, Int. J. Mol. Sci. 9 (2008) 2570–2584 http://doi.org/10.3390/ijms9122570.
[37] M.M. Monte, T. Wang, J.W. Holland, J. Zou, C.J. Secombes, Cloning and characterization of rainbow trout interleukin-17a/F2 (IL-17A/F2) and IL-17 receptor A: expression during infection and bioactivity of recombinant IL-17a/F2, Infect. Immun. 81 (2013) 340–353 https://doi.org/10.1128/IAI.00599-12.
[38] J. Nácher-Mestre, R. Serrano, E. Beltrán, J. Pérez-Sánchez, J. Silva, V. Karalazos, F. Hernández, M.H.G. Berntssen, Occurrence and potential transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients, Chemosphere 128 (2015) 314–320 https://doi.org/10.1016/j.chemosphere.2015.02.021.
[39] T. Nagasawa, C. Nakayasu, A.M. Rieger, D.R. Barreda, T. Somamoto, M. Nakao, Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates, Front. Immunol. 5 (2014), https://doi.org/10.3389/fimmu.2014.00445.
[40] B. Okamura, H. Hartikainen, H. Schmidt-Posthaus, T. Wahli, Life cycle complexity, environmental change and the emerging status of salmonid proliferative kidney disease: PKD as an emerging disease of salmonid fish, Freshw. Biol. 56 (2011) 735–753 https://doi.org/10.1111/j.1365-2427.2010.02465.x.
[41] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal immunity and B cells in teleosts: effect of vaccination and stress, Front. Immunol. 6 (2015), https://doi.org/10.3389/fimmu.2015.00354.
[42] C. Pietsch, Zearalenone (ZEN) and its influence on regulation of gene expression in carp (Cyprinus carpio L.) liver tissue, Toxins 9 (2017) 283 https://doi.org/10.3390/toxins9090283.
[43] C. Pietsch, S. Kersten, H. Valenta, S. Dänicke, C. Schulz, P. Burkhardt-Holm, R. Junge, Effects of dietary exposure to zearalenone (ZEN) on carp (Cyprinus carpio L.), Toxins 7 (2015) 3465–3480 http://doi.org/10.3390/toxins7093465.
[44] C. Pietsch, B.A. Katzenback, E. Garcia-Garcia, C. Schulz, M. Belosevic, P. Burkhardt-Holm, Acute and subchronic effects on immune responses of carp (Cyprinus carpio L.) after exposure to deoxynivalenol (DON) in feed, Mycotoxin Res. 31 (2015) 151–164 https://doi.org/10.1007/s12550-015-0226-6.
[45] C. Pietsch, J. Noser, F.E. Wettstein, P. Burkhardt-Holm, Unraveling the mechanisms involved in zearalenone-mediated toxicity in permanent fish cell cultures, Toxicon 88 (2014) 44–61 http://doi.org/10.1016/j.toxicon.2014.06.005.
[46] C. Pietsch, S. Kersten, P. Burkhardt-Holm, H. Valenta, S. Dänicke, Occurrence of deoxynivalenol and zearalenone in commercial fish feed: an initial study, Toxins 5 (2013) 184–192 http://doi.org/10.3390/toxins5010184.
[47] G.C. Pistol, C. Braicu, M. Motiu, M.A. Gras, D.E. Marin, M. Stancu, L. Calin, F. Israel-Roming, I. Berindan-Neagoe, I. Taranu, Zearalenone mycotoxin affects immune mediators, MAPK signalling molecules, nuclear receptors and genome-wide gene expression in pig spleen, PloS One 10 (2015) e0127503https://doi.org/10.1371/journal.pone.0127503.
[48] R Core Team, R: a Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2017https://www.R-project.org.
[49] I. Rodrigues, L.J. Chin, A comprehensive survey on the occurrence of mycotoxins in maize dried distillers' grain and solubles sourced worldwide, World Mycotoxin J. 5 (2012) 83–88 http://doi.org/10.3920/WMJ2011.1308.
[50] D.L. Roeber, R.C. Cannell, K.E. Belk, R.K. Miller, J.D. Tatum, G.C. Smith, Implant strategies during feeding: impact on carcass grades and consumer acceptability, J. Anim. Sci. 78 (7) (2000) 1867–1874 https://doi.org/10.2527/2000.7871867x.
[51] P. Schwartz, T.D. Bucheli, F.E. Wettstein, P. Burkhardt-Holm, Life-cycle exposure to the estrogenic mycotoxin zearalenone affects zebrafish (Danio rerio) development and reproduction, Environ. Toxicol. 28 (2013) 276–289 http://doi.org/10.1002/tox.20718.
[52] P. Schwartz, K.L. Thorpe, T.D. Bucheli, F.E. Wettstein, P. Burkhardt-Holm, Short-term exposure to the environmentally relevant estrogenic mycotoxin zearalenone impairs reproduction in fish, Sci. Total Environ. 409 (2010) 326–333 http://doi.org/10.1016/j.scitotenv.2010.10.017.
[54] H. Segner, B.M.L. Verburg-van Kemenade, M. Chadzinska, The immunomodulatory role of the hypothalamus-pituitary-gonad axis: proximate mechanism for reproduction-immune trade offs? Developmental & Comparative Immunology, Neuroendocrine-immune system interactions: An evolutionary perspective 66 (2017) 43–60 https://doi.org/10.1016/j.dci.2016.07.004.
[55] Z. Svobodová, D. Pravda, J. Paláčková, Unified methods of haematological examination of fish, Methods 20 (1991) 31 (Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic).
[56] E. Szwejser, B.M.L. Verburg-van Kemenade, M. Maciuszek, M. Chadzinska, Estrogen-dependent seasonal adaptations in the immune response of fish. Hormones and behavior, neuroendocrine-immune interactions: implications for integrative and comparative, Physiol. 88 (2017) 15–24 https://doi.org/10.1016/j.yhbeh.2016.10.007.
[57] A.G.J. Tacon, M.R. Hasan, M. Metian, Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects. FAO Fisheries and Aquaculture Technical Papers 564, Food and Agriculture Organization of the United Nations (2011) 61–62 (Rome).
[58] M.G. Thomas, J.A. Carroll, S.R. Raymond, R.L. Matteri, D.H. Keisler, Transcriptional regulation of pituitary synthesis and secretion of growth hormone in growing wethers and the influence of zeranol on these mechanisms, Domest. Anim. Endocrinol. 18 (2000) 309–324 https://doi.org/10.1016/S0739-7240(00)00052-7.
[59] S.G. Thompson, J.A. Barber, How should cost data in pragmatic randomised trials be analysed? BMJ, Br. Med. J. 320 (2000) 1197 https://doi.org/10.1136/bmj.320.7243.1197.
[60] T. Wang, C.J. Secombes, The cytokine networks of adaptive immunity in fish, Fish Shellfish Immunol. 35 (2013) 1703–1718 https://doi.org/10.1016/j.fsi.2013.08.030.
[61] T. Wang, S.A.M. Martin, C.J. Secombes, Two interleukin-17C-like genes exist in rainbow trout Oncorhynchus mykiss that are differentially expressed and modulated, Dev. Comp. Immunol. 34 (2010) 491–500 https://doi.org/10.1016/j.dci.2009.11.011.
[62] Y.C. Wang, J.L. Deng, S.W. Xu, X. Peng, Z.C. Zuo, H.M. Cui, Y. Wang, Z.H. Ren, Effects of zearalenone on IL-2, IL-6, and IFN-γ mRNA levels in the splenic lymphocytes of chickens, Sci. World J. 2012 (2012) 567327 https://doi.org/10.1100/2012/567327.
[63] J.E. Williams, S.J. Ireland, T.A. Mollett, D.L. Hancock, E.E. Beaver, S. Hannah, Influence of zeranol and breed on growth, composition of gain, and plasma hormone concentrations, J. Anim. Sci. 69 (1991) 1688–1696 https://doi.org/10.2527/1991.6941688x.
[64] M. Woźny, K. Obremski, T. Zalewski, M. Mommens, A. Łakomiak, P. Brzuzan, Transfer of zearalenone to the reproductive system of female rainbow trout spawners: a potential risk for aquaculture and fish consumers? Food Chem. Toxicol. 107 (2017) 386–394 http://doi.org/10.1016/j.fct.2017.07.010.
[65] M. Woźny, S. Dobosz, K. Obremski, P. Hliwa, P. Gomułka, A. Łakomiak, R. Różyński, T. Zalewski, P. Brzuzan, Feed-borne exposure to zearalenone leads to advanced ovarian development and limited histopathological changes in the liver of premarket size rainbow trout, Aquaculture 448 (2015) 71–81 http://doi.org/10.1016/j.aquaculture.2015.05.032.
[66] M. Woźny, K. Obremski, E. Jakimiuk, M. Gusiatin, P. Brzuzan, Zearalenone contamination in rainbow trout farms in north-eastern Poland, Aquaculture 416–417 (2013) 209–211 http://doi.org/10.1016/j.aquaculture.2013.09.030.
[67] A. Zinedine, J.M. Soriano, J.C. Moltó, J. Mañes, Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin, Food Chem. Toxicol. 45 (2007) 1–18 http://doi.org/10.1016/j.fct.2006.07.030.