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BIOLOGICAL EFFECTS OF AFLATOXIN B_1 AND STERIGMATOCYSTIN IN COMMON CARP

PhD thesis

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1 INTRODUCTION AND OBJECTIVES

1.1 Introduction

Aquaculture is currently one of the fastest-growing industries globally and, consequently, requires increasing amounts of feed. The availability of conventional feed materials such as fishmeal and fish oil, however, has not increased with demand, leading to the use of plant proteins as an alternative, which can significantly increase the risk of mycotoxin contamination and mycotoxin load. Although the effects of mycotoxins on fish and various aquatic organisms are currently not well understood, there are a growing number of studies describing a range of pathological and growth problems associated with mycotoxin exposure in various fish species. In our previous experiments we have investigated the effects of other mycotoxins, such as T-2 toxin, as well as deoxynivalenol (DON), ochratoxin A (OTA) and fumonisin B1 (FB1) on the expression of genes responsible for the regulation of certain components of the antioxidant defence system in carp.

Toxic effects induced by aflatoxin B_1 (AFB_1) and sterigmatocystin (STC), mainly produced by *Aspergillus* mold species, are already well known. Still, most are based on results from either *in vitro* experiments or *in vivo,* but most long-term, sublethal mycotoxin exposure.

In addition, the short-term effects of mycotoxins such as aflatoxin B_1 and sterigmatocystin on fish are currently poorly understood, so I chose an economically important species, common carp, for my experiments. Although much is known about aflatoxins, there are still uncertainties in the diagnosis of aflatoxicosis in fish. Early aflatoxicosis in fish is often characterised by liver damage, poor growth, pale gills, and immunosuppression. However, the earliest signs of AFB_1 toxicity include changes in body composition and oxidative stress.

However, it is not yet clear to what extent the two mycotoxins induce oxidative stress in fish and what order and to what extent they induce molecular markers of the antioxidant defence system.

It is also unknown how the body's antioxidant defence system reacts after feeding feed contaminated with various mycotoxins, such as AFB_1 and STC. For mycotoxins that are routinely tested, the current regulations are based on data from toxicological studies that did not consider the combined effects of mycotoxins but only one

mycotoxin exposure. However, once in the body, the different mycotoxins may interact with each other and consequently exert antagonistic or synergistic, and/or additive effects, especially if they have a similar mechanism of action and act on the same organ.

1.2 Objectives

1. The main objective of my studies was to investigate the short-term effects of different doses of AFB_1 and STC produced by *Aspergillus* species on the expression of genes encoding the elements of the glutathione redox system and transcription factors responsible for their regulation, following a single exposure.

2. The aim of the experiments was also to assess the direction and extent of the short-term effects of the combined application of AFB_1 and STC after a single exposure on the expression of the elements of the carp glutathione redox system and the genes encoding transcription factors responsible for its regulation.

The following experiments were designed, and parameters were measured to achieve the objectives:
I. Evaluation of short term effects (24 hours) of single, three sublethal doses of aflatoxin in the liver of one-year-old common carp;
II. Evaluation of short term effects (24 hours) of single, three sublethal doses of sterigmatocystin in the liver of one-year-old common carp;
III. Evaluation of short-term effects (24 hours) of a single, sublethal dose of aflatoxin and sterigmatocystin combination in the liver of one-year-old common carp.

Experiments were performed to investigate the expression of genes encoding Nrf2/Keap1-ARE (*kelch-like ECH-associated protein 1/ nuclear factor E2-related factor 2/antioxidant response element*) pathway (*Keap1* and *Nrf2*), the phospholipid hydroperoxide glutathione peroxidase enzyme (glutathione peroxidase 4a and glutathione peroxidase 4b (*gpx4a* and *gpx4b*)) and genes encoding glutathione synthetase (*gs*) and glutathione reductase (*gr*).

2 MATERIALS AND METHODS

2.1 Mycotoxin production and artificial contamination of feed

2.1.1 Artificial mycotoxin contamination of the feed

Aflatoxin was produced by an *Aspergillus flavus* (ZT80) strain in corn substrate in collaboration with the Department of Environmental Safety of Hungarian University of Agriculture and Life Sciences, while high purity (99.0%) sterigmatocystin was obtained from Romer Labs (Tulln, Austria).

In the experiment with carp, a commercially available Aqua Garant Classic™ feed was used, ground, and mixed with corn substrate with known concentrations of aflatoxin B_1. For the groups treated with sterigmatocystin, the sterigmatocystin was dissolved in ethanol and then sprayed uniformly onto the pelleted feed in the amount required for the concentration to be tested. The doses of each treatment group were diluted 1:4 with distilled water immediately before use to be administered through gavage directly into the gut. As a control, a 1:4 aqueous mixture of the basal feed was used. The aqueous suspension of the feeds was administered using a pipette.

The aflatoxin content of control and artificially contaminated feeds was determined by high-resolution liquid chromatography after immunoaffinity pre-treatment, fluorimetric detection, while sterigmatocystin determination was performed by the HPLC-UV method.

2.2 Experimental protocols and sampling method

At the start of the experiment, the artificially contaminated and control diet was applied by gavage directly into the gut once. Liver samples were taken from 6 randomly selected carp specimens before the start of the trial (absolute control: 0 h) and 6 randomly selected fishes in each group at every 8th hour during a 24-h period. All samples were taken into 1.5 ml collection tubes, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

2.3 Gene expression

2.3.1 RNA purification and reverse transcription

Total RNA was purified from liver samples of experimental animals using NucleoZOL reagent, and genomic DNA contamination was removed by DNase I treatment. Pools of RNA were generated per treatment group, with equal amounts of RNA per individual (n=6),

and 1000 ng of pooled RNA was reverse transcribed into cDNA using a random nonamer (9-mer) for qPCR measurements.

2.3.2 Real-time PCR measurements

Expression of glutathione peroxidase 4a and b (*gpx4a* and *gpx4b*), glutathione synthetase (*gs*), and glutathione reductase (*gr*), Nrf2 (*nrf2*), Keap1 (*keap1*) target genes, and the β-actin endogenous control gene, was determined by quantitative real-time PCR using SYBRGreen. The primers used for the quantification of the mRNA transcriptional levels of the target and endogenous control gene were chosen based on the literature. Measurements were performed using the Step One Plus™ Real-Time PCR systems using Maxima SYBRGreen qPCR Master Mix in 5 replicates. No-template controls were also performed for each primer pair. The PCR profile for the *gpx4a* and *gpx4b* target genes consisted of 95 °C for 10 min for preamplification denaturation (PAD), and 95 °C 15s, 55 °C 30 s and 72 °C 30 s for 45 cycles, for *nrf2* and *keap1* target genes 95 °C for 10 min PAD, and 95 °C 15s, 60 °C 30 s and 72 °C 30 s for 45 cycles and for *gs* and *gr* target genes 95 °C for 10 min PAD, and 95 °C 15s, 56,5 °C and 72 °C 30 s for 50 cycles where SYBR Green signal was detected at the end of the extension period.

2.3.3 Real-time PCR evaluation and calculation

The amplified products were verified by melting curve analysis and gel electrophoresis. The threshold cycle (Ct) of the target genes (*gpx4a*, *gpx4b*, *nrf2*, *keap1*, *gs* and *gr*) and the endogenous control gene (b-actin), was determined by StepOne™/StepOnePlus™ Software v2.2, the delta Ct values (ΔCt), delta-delta Ct values (ΔΔCt) and relative quantification ($RQ = 2^{-\Delta\Delta Ct}$) values were calculated.

2.4 Statistical analysis

The statistical evaluation of the experimental results was performed using descriptive statistical calculations, one-way and two-way analysis of variance (ANOVA). The normal distribution of the results was checked by the Kolmogorov-Smirnov test, and the homogeneity of variance was checked by the Bartlett test. The calculations were performed using GraphPad Prism 7.0 software.

3 RESULTS AND DISCUSSION

3.1 Summary of short term effect of aflatoxin B_1

In this experiment, the effects of three different doses of aflatoxin B_1 were investigated on the parameters of the glutathione redox system and the expression of genes responsible for its regulation.

From our previous experiments, it was known that at a water temperature of 19 ± 1 °C, the average transit time of the feed for the carp weight range used in the experiment was 16 hours. Therefore, this is the time period that determines the time of bioavailability of mycotoxins and their absorption from the intestinal tract.

The doses used in the experiment (Table 1) were based on the maximum AFB_1 content (20 μg/kg feed (574/2011/EC)) set by the European Union for feed materials.

Table 1: Doses of aflatoxin B_1 used in the experiment in each group (μg/kg body weight)

Treatnent group	Control	A1 (low)	A2 (medium)	A3 (high)
AF	<1.0	1.46	2.92	5.85

No mortality or sublethal symptoms were observed in any experimental groups during the 24-h experiment, presumably due to the relatively low dose, as the *per os* LD_{50} for rainbow trout, which is a particularly sensitive species to AFB_1, is 500 μg/kg body weight.

Changes in the gene expression level of *keap1* and *nrf2* involved in the Keap1-Nrf2 regulatory pathway showed a prolonged and fluctuating effect. The *keap1* gene showed an initial dose-dependent decrease, followed by a subsequent inhibition of gene expression at 16h while no significant differences were observed at 24h in response to the doses applied. In the case of *nrf2* gene expression, the shift was even more pronounced, as there was no change in the initial values measured at hour 8 for either dose. However, at hour 16, reduced gene expression values were observed in groups A2 and A3 compared to the control. At 24 hours, there was no significant difference for *keap1* compared to the control, while for *nrf2*, only as effect of A1 and A3 showed an increase. These differences, taking into account the sub-lethal doses used in the experiment and the degree of oxidative stress they induce, can be explained by the hierarchical model of oxidative stress. However, *nrf2* transcription

may also be affected by other transcription pathways. For example, the promoter region of the *nrf2* gene contains an ARE (Antioxidant Response Element) sequence, which allows Nrf2 to directly activate its transcription, providing a positive feedback mechanism to amplify Nrf2 effects. In addition, Nrf2 transcription is regulated by several other transcription factors, such as the aryl hydrocarbon receptor (AhR).

The expression of genes encoding elements of the glutathione redox system (*gpx4a* and *gpx4b*), after initial inhibition, showed a prolonged increase compared to the control, which appeared after 16 h of sampling. In some cases, the increase was only tendentious. However, the expression of the *nrf2* gene remained unchanged at 8 h after the doses were applied, whereas it decreased at 16 h. This suggests that the level of oxidative stress induced by AFB_1 exposure, based on the hierarchical oxidative stress model, has either exceeded a threshold, which no longer primarily results in the activation of the Nrf2-ARE pathway or the doses applied have not yet induced such a level of oxidative stress that is also manifested at the level of expression of the genes investigated.

The short-term effects described in the present experiment cannot be directly compared with previous literature data, as these are not available for the carp species.

AFB_1 treatment resulted in significant differences in sampling time within each experimental group for all genes tested. These changes were detectable in both mycotoxin-treated and control groups. This is presumably due to a single dose of feed administered through the gavage but may also be due to unknown factors and biological circadian rhythms. The light-dark cycle is a key factor influencing the circadian rhythm of fish.

In this context, it is important to point out that although the 12:12 hour light schedule that was used deviated from the natural rhythm, the effect was the same in all test groups. However, the circadian rhythm could have been disrupted by the light program used, which could have affected some parameters of the antioxidant enzymes and membrane transporters at both mRNA and protein levels.

In the experiment, the combined effect of treatment x time in the case of each gene was statistically confirmed, suggesting that not only the dose administered but also the time elapsed since treatment, including the circadian rhythm, may play a key role in the effects

produced. In a previous study with in vitro cell lines, AFB_1 has also been described to show not only dose-dependent but also time-dependent changes.

3.2 Summary of short term effect of sterigmatocystin

There is currently little data available on STC contamination in feed. The European Union does not yet regulate its levels in feed. For this reason, ten times the doses used in the aflatoxin B_1 experiment were used. Indeed, the toxicity of STC has been estimated to be 1/10 of AFB_1 in several publications. Doses per body weight are shown in Table 2.

Table 2. Mycotoxin doses used in the experiment in each group (μg/kg body weight)

Treatment group	Control	S1 (low)	S2 (medium)	S3 (high)
STC	<1.0	18.89	36.95	72.70

For carp, an LD_{50} of 211 μg/kg feed has been reported in the literature. However, no mortality was observed in any of the experimental groups over the 24-hour duration of the experiment, although the doses applied were 5x, 10x and 20x the LD_{50}. This may be due to the lower toxicity of STC compared to AFB_1.

The relative gene expression of *nrf2* and *keap1* showed strong induction at 16 and 24 h. For *keap1*, I observed induction in group S2 at all sampling time points, while in groups S1 and S3 I observed both induction and control levels at different sampling time points. For *nrf2*, gene expression levels decreased in the S1 group at 8 h, were unchanged in the S2 and S3 groups compared to the control, while significant induction was detected as effect of all doses at 16 and 24 h. In the case of *nrf2*, it can be clearly seen that initial inhibition in the S1 group at 8 h sampling, and near control values in the S2 and S3 groups, were followed by strong induction at 16 and 24 h. *Keap1* gene expression showed induction in S1 and S2 treatment groups as early as the 8th hour of sampling, returning to near control levels by 24 hours at the lowest dose.

Based on the hierarchical oxidative stress model, it can be assumed that mild oxidative stress was induced by the doses applied. The model suggests that under low oxidative stress, the Nrf2 protein activates the gene expression of antioxidant enzymes. In contrast, under higher ROS formation, the Nrf2-Keap1 pathway is no longer activated, requiring Nrf2-inhibitory activity of Keap1.

I have not had the opportunity to compare my results with those of previous experiments with fish, as they are not available in the literature. However, a 24-hour in vivo experiment with rats confirmed my results in that moderate oxidative stress was also observed when animals were treated with STC at doses of 10, 20, and 40 mg/kg body weight.

The expression of phospholipid hydroperoxide glutathione peroxidase genes (*gpx4a* and *gpx4b*) has a prominent antioxidant role in fish after initial inhibition showed a strong induction at 16 and 24 h of sampling, similar to the expression changes of *nrf2* in S1 and S3 groups, after initial inhibition, showed a strong induction at 16 and 24 h of sampling compared to the control, while for *gpx4b* gene, induction at low dose was observed as early as 8 h of sampling.

Similar changes were observed for the glutathione synthetase (*gs*) gene as for *gpx4a* gene mentioned above. Significant inhibition of gene expression was detected in the S1 group at the initial 8 h sampling, while significant induction was observed in all three STC-contaminated feed groups at 16 and 24 h. However, the dose-dependent evolution of the measured values was only observed at hour 16. In contrast, the glutathione reductase (*gr*) gene showed significant inhibition in S1 and S3 groups at the initial 8 h sampling followed by induction at 16 h. Subsequently, however, by the 24th hour, gene expression levels had returned to control levels.

The results suggest that mild oxidative stress was induced by the applied doses because the expression of *nrf2* and its regulated genes was induced at 16 and 24 h after 8 h of inhibition.

Significant differences in sampling times were also observed within each experimental group as a result of STC treatment. However, these changes were detectable in both mycotoxin-treated and control groups. This is presumably due to a single dose of feed administered through the gavage but may also be due to unknown factors and biological circadian rhythms.

The combined effect of treatment x time for all genes was also statistically supported in this experiment, showing that not only the dose applied but also the time since treatment plays a key role in the effects that develop. Both dose- and time-dependent changes were also observed for STC in an *in vitro* experiment with human neuroblastoma cells (SH-SY5Y).

3.3 Summary of short term combined effect of aflatoxin B_1 and sterigmatocystin

The effects of co-contaminated feed with various mycotoxins are poorly understood, especially for fish. There is little information in the literature on the combined effects of aflatoxin and sterigmatocystin. In the third experiment, the effects of aflatoxin and sterigmatocystin, individually and in combination, were investigated on the expression of some parameters of the glutathione redox system and the genes responsible for their regulation. The mycotoxin content of the feed used for the treatment is presented in Table 3. The doses used were selected based on the previous two experiments. No mortality was observed during the 24-hour duration of the experiment.

Table 3. Mycotoxin doses used in the experiment in each group (μg/kg body weight)

Treatment group	Control	AFB_1	STC	AFB_1+STC
Aflatoxin (AFB_1)	<1.0	0.95	-	0.95
Sterigmato-cystin (STC)	<1.0	-	10.27	10.27

The results show that the combination of AFB_1 and STC has a synergistic effect on *nrf2* gene expression. AFB_1 induction at 8 h was followed by inhibition at 16 and 24 h sampling. In an experiment with broiler chickens, AFB_1 exposure also resulted in inhibition of *nrf2* at both mRNA and protein levels and reduced expression of xenobiotic transformation phase II genes such as glutathione-S transferase (*gst*). Expression of the *keap1* gene showed a dual response in this study. Inhibition was observed at 8 and 16 h sampling and induction at 24 h sampling in the group co-treated with aflatoxin and sterigmatocystin, confirming a synergistic effect between AFB_1 and STC.

The expression of the *nrf2* gene, as a result of the applied doses, changed at 8 h only in the group treated with aflatoxin and the two mycotoxins in combination, while at 16 and 24 h, decreased values were observed in the group treated with aflatoxin and sterigmatocystin. This suggests that the applied doses have not yet caused the degree of oxidative stress that was also manifested at the expression of the genes studied.

The expression of the phospholipid hydroperoxide glutathione peroxidase genes (*gpx4a* and *gpx4b*) increased or decreased at each sampling time point. For *gpx4a*, a gradual decrease in gene expression was observed in the aflatoxin-treated group, whereas for *gpx4b*, a continuous induction of gene expression was observed compared to the control. However, in the STC group, the initial inhibition at 8 and 16 h was followed by induction at 24 h for the *gpx4a* gene, while *gpx4b* gene expression decreased at 16 and 24 h sampling compared to the control. In the AFB_1+STC group, however, the *gpx4a* gene showed inhibition at all three sampling time points, while the *gpx4b* gene showed induction at 8 and 24 h.

The expression of glutathione synthetase and glutathione reductase genes was similarly altered. At the initial sampling time of 8 h, induction was observed in the groups treated with aflatoxin and in the groups treated with aflatoxin and sterigmatocystin together, followed by varying degrees of inhibition at 16 and 24 h. The expression of the *nrf2* gene in the aflatoxin and sterigmatocystin-treated groups was at or below control levels during the 24-h experiment, which may explain the low expression of the antioxidant genes tested.

AFB_1+STC treatment resulted in significant differences in the genes tested within experimental groups and between sampling time points. However, these changes were detectable in both mycotoxin-treated and control groups. This is presumably due to a single dose of feed administered through the gavage but may also be due to unknown factors and biological circadian rhythms.

In this experiment, I was also able to statistically confirm the treatment x time effect for all the genes tested, showing that not only the dose but also the time since treatment plays a key role in the effects that occur.

4 CONCLUSIONS AND SUGGESTIONS

4.1 Conclusions

The primary objective of my studies was to assess the detectable changes in the expression of genes encoding and regulating elements of the glutathione redox system following AFB_1 and STC exposure in carp over a 24-h period. My aim was also to assess the order and extent of changes in these markers when the two mycotoxins are applied together. In the experiments, artificially contaminated feed containing known mycotoxins and control feed containing mycotoxins below the limit of detection was introduced into the animals in a single dose via gavage directly into the gut.

In all three experiments, the expression changes of *nrf2*, *keap1*, and *gpx4a* and *gpx4b*, encoding the antioxidant enzymes GPx4, which are of major importance in fish, as well as *gs* and *gr*, encoding enzymes involved in the synthesis and subsequent reduction of the GPx4 co-substrate, reduced glutathione, were measured.

The three experiments took place in the autumn, three years in a row. The body weights of the animals, which at the start of the experiment were 34.20 ± 3.99 g in the experiment with AFB_1, 27.09 ± 5.38 g in the experiment with STC and 49.07 ± 8.85 g in the experiment with AFB_1 and STC, were always dependent on the actual harvest.

Accordingly the doses used in the aflatoxin B_1 experiment were 1.46; 2.92; and 5.85 μg/kg body weight; in the sterigmatocystin experiment were 18.89; 36.95; and 72.70 μg/kg body weight and in the third experiment were 0.95 μg AFB_1/kg; 10.27 μg STC/kg; and 0.95 μg AFB_1/kg and 10.27 μg STC/kg body weight.

It is important to underline that the lower doses used for AFB_1 (93.5 and 187.1 μg/kg feed) can occur in practice in extreme cases. To the best of our knowledge, the STC doses used are not found in practice (1, 2, or 4 mg/kg feed).

According to a global survey carried out in 2019, the aflatoxin contamination in compound feed in Europe was 8%, with an average contamination of 10 μg/kg feed and a maximum of 237 μg/kg feed. However, little data is currently available on the STC content of the feed. It is also important to note that the prevalence of multi-mycotoxins in the 2019 survey was as follows: an average of 34 mycotoxins and metabolites per sample, and 90% of the samples

tested contained at least 10 mycotoxins and metabolites. This clearly shows that assessing the impact of multi-mycotoxin exposure is an extremely important task.

There is currently little data available on the susceptibility of fish to certain mycotoxins. Among the fish species studied so far, the following order of susceptibility to aflatoxin has been established: rainbow trout>mosquitofish>coho salmon>channel catfish>rohu>nile tilapia. The differences in sensitivity between fish species are significant, presumably due to differences in the activity and gene expression of enzymes involved in the metabolism of AFB_1, and STC, resulting in an imbalance between phase I and phase II biotransformation due to its similar effect.

No mortality or sub-lethal symptoms were observed in any of the experiments at the doses applied, although the STC doses applied were higher than the LD_{50} of 211 µg STC/kg feed for carp. For AFB_1, no *per os* LD_{50} values for carp species were found in the literature.

When exposed to aflatoxin B_1 and sterigmatocystin alone, a dual response in the expression of the genes tested was observed at the doses applied. Initial inhibition at 8 h was followed by induction by 16 h, which may be related to the transit time of the feed, i.e., the time required for the absorption of the feed artificially contaminated with mycotoxins from the intestinal tract, followed by the transport of mycotoxins to the liver and finally the time required for effect to be exerted.

The expression of genes encoding the Nrf2-Keap1 pathway (*nrf2* and *keap1*), which regulates gene expression of antioxidant enzymes at the transcriptional level, showed a higher inhibition upon higher doses of aflatoxin B_1 exposure. As a result, *keap1* gene expression was lower than control at 8h for all three doses (A1, A2, A3), and remained so at 16h for the highest dose, but was not significantly higher than control at 24h, while at 8h it was lower than control as effect of the lowest dose used in my experiments (0.95 µg/kg b.w.). The expression of *nrf2* gene was higher than the control as effect of the lowest dose (0.95 µg/kg ttm) at the 8h of sampling, while at the 16th hour it was lower as effect of the lowest (0.95 µg/kg ttm), medium and highest doses (A2, A3), in contrast to the 24th hour (0.95 µg/kg ttm), whereas at 24h it was lower at the lowest AFB_1 dose (0.95 µg/kg ttm) and higher at the low (A1) and highest (A3) doses than the control.

In contrast, inhibition by STC was found to be less pronounced, as *keap1* gene expression increased at 8h for low and medium doses (S1, S2) and then also exceeded the control at 24 h, except for low dose (S1). However, as effect of the lowest dose (10.27 µg/kg ttm), induction was observed at the initial sampling time of 8 h. The expression of *nrf2* gene as effect of the lowest dose (10.27 µg/kg b.w.) did not change in the first 16 h after treatment compared to control values. However, its expression was lower for the lowest dose (10.27 µg/kg b.w.) at the 24th hour sampling and for the low dose (S1) at the 8th hour sampling, whereas it was higher than the control at the 16th and 24th hours for all three doses applied (S1, S2 and S3). As effect of the AFB_1 and STC combination, *nrf2* gene expression was higher than the control at all three sampling time points (8, 16 and 24 h), while *keap1* gene expression was higher than the control at 16 and 24 h.

Based on these results, it can be assumed that the oxidative stress level induced by AFB_1 either exceeded a threshold value, according to the hierarchical oxidative stress model, which no longer primarily activates the Nrf2-ARE pathway or the doses used have not yet resulted in oxidative stress to the extent that is reflected in the expression of the genes studied. However, the Nrf2-ARE pathway was activated by higher doses of STC, so that STC, or reactive metabolites formed from it, are more effective in the liver in inducing ROS formation or doses of STC ~10x higher than those used in the aflatoxin experiment induce mild oxidative stress. Co-treatment with AFB_1 and STC activated *nrf2*, so the combined effect of the two mycotoxins could be synergistic, i.e., the reactive metabolites they produced were more effective in inducing ROS formation than when they were applied individually.

Significant differences in the genes involved in regulating the glutathione redox system were also observed in response to mycotoxin stress. Expression of the *gpx4a* gene was lower at 16 and 24 h after AFB_1 loading at low doses (A1) and significantly higher at higher doses (A2, A3), while as effect of the lowest dose (0.95 µg/kg b.w.) it was lower than control, whereas expression of the *gpx4b* gene increased significantly at 16 and 24 h as effect of all doses applied. The expression of the *gs* gene was higher as effect of the lowest dose (0.95 µg/kg b.w.) and the low (A1) dose at 8h, at 16h as effect of all three doses applied (A1, A2 and A3) and as effect of the low (A1) and high (A3) doses at 24 h when compared to control. The expression of the *gr* gene exceeded the control value by the 8h hour at the lowest dose (0.95 µg/kg b.w.) and by the 16th

hour at the low dose (A1). In the STC experiment, the induction after initial inhibition was more pronounced for the above-mentioned genes (*gpx4a*, *gpx4b*, *gs* and *gr*) than for AFB_1. This was probably due to the higher dose range used than in the AFB_1 experiment. When AFB_1 and STC were administered together, the expression of the gs and gr genes showed inhibition at 24 h after the initial induction at 8 h. This could be due to lower concentrations of the two mycotoxins compared to their use alone. This is because the lower amount is presumably absorbed from the intestinal tract more rapidly, but is also metabolized more rapidly, and thus may increase the amount of reactive oxygen species more rapidly. However, this presumably reduced ROS production was effectively neutralised by the liver by 16 and 24 hours due to the efficient expression of genes encoding antioxidant enzymes.

4.2 Suggestions

In recent years, an increasing number of studies in the literature attributes the toxicity of certain mycotoxins to the formation of free radicals and the consequent induction of oxidative stress. I, therefore, propose to continue the research to monitor the expression changes of the genes I have studied for other mycotoxins with different chemical structures.

Due to the structure of AFB_1 and STC and to the transcriptional activation CYP450 genes, both mycotoxins are putative aryl hydrocarbon receptor agonists; therefore, I propose to continue the research using new target genes and to investigate the aryl hydrocarbon receptor (AhR)-Xenobiotic Response Element (XRE) pathway, also for other mycotoxins with different chemical structures but also inducing CYP450 genes.

Finally, I propose to extend the experiments to investigate how mycotoxins may induce post-transcriptional changes in different miRNAs regulating Nrf2.

5 NEW SCIENTIFIC RESULTS

Evaluating the effects of the applied doses of mycotoxins on common carp in the observed 24 hour period, I concluded that:

1. Different doses (1.46, 2.92 and 5.85 g/kg b.w.) of single per os **aflatoxin B_1** loading in the liver of common carp juveniles resulted that:

The expression of ***keap1*** and ***nrf2*** genes was activated after initial inhibition. *Keap1* gene expression was lower than control at 8h for all three doses, and persisted at 16h for the highest dose, but did not significantly exceed it at 24h. The *nrf2* gene expression was lower at 16h for the medium and highest doses, and higher at 24h for the lowest and highest doses.

The expression of the ***gpx4a*** and ***gpx4b*** genes was initially induced in a dose-dependent manner after the inhibition observed at the highest dose, which correlates with the activation of the Keap1-Nrf2-ARE pathway. Expression of gpx4a as effect of the applied doses were higher than the control at 16 and 24h sampling, while the expression of *gpx4b* gene increased significantly at 16 and 24 h after all three doses.

Epression of the ***gs*** gene was induced as effect of all doses applied by the 16h. Expression of the gr gene was induced by the low dose at the 16th hour after the initial inhibition at the medium and high doses. Thus, activation of the Keap1-Nrf2-ARE pathway can be assumed here as well. Expression of the ***gs*** gene was higher than control at the 8th hour at the lowest dose and at the 16th and 24th hours at the lowest and highest doses, respectively. ***Gr*** gene expression was also higher than the control at the lowest dose at hour 16.

2. Different doses (18,89, 36,95 és 72,70 µg/kg b.w.) of single per os **STC** loading in the liver of common carp juveniles resulted that:

There was an initial inhibition of ***nrf2*** gene expression at low doses, followed by activation at all doses applied, but the initial inhibition was shorter than for AFB_1. Expression of the ***keap1*** gene increased at the 8th hour for low and medium doses, and then also exceeded the control at the 24th hour except for the lowest dose. The expression of *nrf2* gene was lower than the control at 8 h for the

lowest dose and higher than the control at 16 h and 24 h for all doses applied.

The expression of the ***gpx4a*** gene was induced after initial inhibition as effect of low and high doses, which was more pronounced than for AFB_1 and correlated with activation of the Keap1-Nrf2-ARE pathway. Expression of the *gpx4a* gene was lower than control at 8h as effect of the lowest and highest doses and higher than control at 16 and 24h for all doses applied. Expression of the ***gpx4b*** gene was higher than the control at the low dose at 8 h and then at 16 and 24 h for all doses applied.

The ***gs*** and ***gr*** genes were induced after the initial inhibition, which was more pronounced compared to AFB_1 and here also the activation of the Keap1-Nrf2-ARE pathway is assumed. The expression of the *gs* gene was lower than the control at the lowest dose at 8h and then higher than the control at 16h and 24h for all doses applied. Expression of the *gr* gene was lower than the control at the 8th hour for the lowest and highest doses and higher than the control at the 16h hour for all doses applied.

3. A single per os **co-exposure to AFB_1 and STC** at doses of 0.95 and 10.27 μg/kg body weight respectively resulted that:

The ***nrf2*** gene was activated to a greater extent than when the two mycotoxins were applied alone, suggesting a synergistic effect between them, i.e. they were more effective in affecting the expression of redox-sensitive genes during co-exposure. The expression of the *keap1* gene exceeded that of the control at 16 and 24h, and the expression of the *nrf2* gene exceeded that of the control at all three sampling time points (8, 16 and 24 h).

The expression of the ***gpx4a*** gene was less activated by co-expression. The expression of *gpx4a* was inhibited at all three sampling time points, whereas the expression of *gpx4b* increased at 8 and 24 h.

The expression of the ***gs*** and ***gr*** genes showed a moderate inhibition at the 24th hour of sampling after the initial activation at 8 h, which also suggests a synergistic effect between the mycotoxins used.

PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS

Publications in scientific journals:

Kövesi B, Balogh K, Pelyhe Cs, Mézes M 2017 A szterigmatocisztin mikotoxin toxikus hatásai az állati szervezetre – irodalmi áttekintés. Magyar Állatorvosok Lapja; 139(7), 427-432. [IF:0.196]

Kövesi B, Pelyhe Cs, Zándoki E, Mézes M, Balogh K 2018 Changes of lipid peroxidation and glutathione redox system, and expression of glutathione peroxidase regulatory genes as effect of short-term aflatoxin B1 exposure in common carp. Toxicon, 144, 103–108. [IF:2.276]

Kövesi B, Pelyhe Cs, Zándoki E, Miklós M, Balogh K 2019 Effect of short-term sterigmatocystin exposure on lipid peroxidation and glutathione redox system and expression of glutathione redox system regulatory genes in common carp liver. Toxicon, 161, 50-56. [IF: 2.201]

Kövesi B, Kulcsár S, Ancsin Z, Zándoki E, Erdélyi M, Mézes M, Balogh K 2021 Individual and combined effects of aflatoxin B1 and sterigmatocystin on lipid peroxidation and glutathione redox system of common carp liver. Toxins, 13(2), 109. [IF: 3.531]

Conference abstracts and proceedings:

Kövesi B, Pelyhe Cs, Balogh K, Mézes M 2016 A szterigmatocisztin biológiai hatásai brojlercsirkében és pontyban. LVIII. Georgikon Napok. Keszthely, p. 95 (ISBN:978-963-9639-84-3)

Kövesi B, Pelyhe Cs, Zándoki E, Balogh K, Mézes M 2017 Aflatoxin B1 rövidtávú hatása az antioxidáns védőrendszer szabályozására pontyban. LIX. Georgikon Napok. Keszthely, p. 114 (ISBN 978-963-9639-88-1)

Kövesi B, Pelyhe Cs, Zándoki E, Balogh K, Mézes M 2017 Aflatoxin B1 hatása a ponty antioxidáns védőrendszerének szabályozására rövidtávú toxinexpozíciót követően; Magyar Szabadgyök-kutató Társaság IX. kongresszusa, Gödöllő, p. 30.

Kövesi B, Pelyhe Cs, Zándoki E, Balogh K, Mézes M 2017 Investigation of the effects of aflatoxin B1 on parameters of the antioxidant defence system and lipid peroxidation in common carp; 6th Scientific day of animal breeding in Gödöllő, International Conference; Gödöllő

Kövesi B, Pelyhe Cs, Zándoki E, Balogh K, Mézes M 2018 Szterigmatocisztin rövidtávú hatásának vizsgálata az antioxidáns védőrendszer szabályozására pontyban. LX. Georgikon Napok. Keszthely, p. 90 (ISBN 978-963-9639-91-1)

Kövesi B, Pelyhe Cs, Zándoki E, Balogh K, Miklós M 2018 Short term effects of sterigmatocystin on the regulation of the antioxidant system in common carp; FIBOK2018, Budapest, p. 71.

Kövesi B, Pelyhe Cs, Zándoki E, Mézes M, Balogh K 2018 Szterigmatocisztin hatása az antioxidáns rendszer szabályozására pontyban rövidtávú toxinexpozíciót követően. Magyar Toxikológusok Társasága TOX'2018 Konferencia, Lillafüred,

Kövesi B, Zándoki E, Balogh K, Mézes M 2019 Investigation of short-term multi-mycotoxin effects on the regulation of the antioxidant system in common carp (Cyprinus Carpio L.); 41st Mycotoxin Workshop, Lisbon, Portugal

Book chapter:

Kulcsár Sz, **Kövesi B**, Mézes M 2019 Aflatoxins, oxidative stress and regulation of glutathione redox system In: Kintzios, S., Mavrikou, S. (eds.) Aflatoxins: Biochemistry, Toxicology, Public Health, Policies and Modern Methods of Analysis. Hauppauge (NY), USA, Nova Science Publishers, pp. 29-50..

Kövesi B, Kulcsár Sz, Balogh K 2020 Egyes mikotoxinok által előidézett oxidatív stressz és annak hatásai az antioxidáns rendszerre, valamint annak szabályozására pontyban (Cyprinus carpio L.) In: Poór P. , Mézes M., Blázovics A. (szerk.) Oxidatív stressz és antioxidáns védekezés a növényvilágtól a klinikumig, Budapest, Magyar Szabadgyök-Kutató Társaság, 146-151.

PUBLICATIONS NOT RELATED DIRECTLY TO THE TOPIC OF THE THESIS

Publications in scientific journals:

Pelyhe Cs, **Kövesi B,** Zándoki E, Kovács B, Szabó-Fodor J, Mézes M, Balogh K 2016 Effect of 4-week feeding of deoxynivalenol- or T-2-toxin-contaminated diet on lipid peroxidation and glutathione redox system in the hepatopancreas of common carp (Cyprinus carpio L.) Mycotoxin Research, 32(2), 77–83.

Pelyhe Cs, **Kövesi B,** Zándoki E, Kovács B, Szabó-Fodor J, Mézes M, Balogh K 2016 Short-term effects of T-2 toxin or deoxynivalenol on lipid peroxidation and the glutathione system in common carp; Acta Veterinaria Hungarica, 64(4), 449–466.

Erdélyi M, Balogh K, Pelyhe Cs, **Kövesi B**, Nakade M, Zándoki E, Mézes M, Kovács B 2018 Changes in the regulation and activity of glutathione redox system, and lipid peroxidation processes in short-term aflatoxin B1 exposure in liver of laying hens; Journal of Animal Physiology and Animal Nutrition, 102(4), 947–952.

Nakade M, Pelyhe Cs, **Kövesi B**, Balogh K, Kovács B, Szabó-Fodor J, Zándoki E, Mézes M, Erdélyi M 2018 Short-term effects of T-2 toxin or deoxynivalenol on glutathione status and expression of its regulatory genes in chicken; Acta Veterinaria Hungarica, 66(1), 28–39.

Pelyhe Cs, **Kövesi B**, Szabó-Fodor J, Zándoki E, Erdélyi M, Kovács B, Mézes M, Balogh K 2018 Age-dependent effects of short-term exposure of T-2 toxin or deoxynivalenol on lipid peroxidation and glutathione redox system in broiler chickens; World Mycotoxin Journal 11(4), 611-624.

Pelyhe Cs, **Kövesi B**, Zándoki E, Kovács B, Erdélyi M, Kulcsár S, Mézes M, Balogh K 2018 Multi-trichothecene mycotoxin exposure activates glutathione-redox system in broiler chicken; Toxicon, 153, 53-57.

Kövesi B, Cserháti M, Erdélyi M, Zándoki E, Mézes M, Balogh K 2019 Long-term effects of ochratoxin A on the glutathione redox system and its regulation in chicken; Antioxidants. *8(*6), 178.

Balogh K, **Kövesi B,** Zándoki E, Kulcsár Sz, Ancsin Zs, Erdélyi M, Dobolyi Cs, Bata-Vidács I, Inotai K, Szekeres A, Mézes M, Kukolya J 2019 Effect of sterigmatocystin or aflatoxin contaminated feed on lipid peroxidation and glutathione redox system and expression of glutathione redox system regulatory genes in broiler chicken; Antioxidants. 8(7), 201.

Kövesi B, Cserháti M, Erdélyi M, Zándoki E, Mézes M, Balogh K 2020 Lack of dose- and time-dependent effects of aflatoxin B1 on gene expression and enzymes associated with lipid peroxidation and the glutathione redox system in chicken; Toxins, 12(2), 84.

Kövesi B, Kulcsár Sz, Zándoki E, Szabó-Fodor J, Mézes M, Balogh K, Ancsin Zs, Pelyhe Cs 2020 Short-term effects of deoxynivalenol, T-2 toxin, fumonisin B1 or ochratoxin on lipid peroxidation and glutathione redox system and its regulatory genes in common carp (Cyprinus carpio L.) liver; Fish Physiology and Biochemistry, 46(6), 1921–1932.

Kövesi B, Kulcsár Sz, Cserháti M, Erdélyi M, Ancsin Zs, Zándoki E, Mézes M, Balogh K, 2021 Modification of the effects of aflatoxin B1 on the glutathione system and its regulatory genes by zeolite; Acta Veterinaria Hungarica. online first doi: 10.1556/004.2021.00002

Abstracts in scientific journals:

Kulcsár Sz, **Kövesi B,** Mézes M, Balogh K, Zándoki E 2019 The Fusarium mycotoxins effect on glutathione system in broiler chicken, Toxicology Letters; 314 pp. S113-S114. , 2 p. /conf. 55th Congress of the European-Societies-of-Toxicology (EUROTOX) - Toxicology - Science Providing Solutions

Conference abstracts and proceedings:

Pelyhe Cs, **Kövesi B**, Zándoki E, Mézes M, Balogh K, Kovács B 2016, Combined effect of T-2 toxin and deoxynivalenol on regulation of the glutathione redox system in broiler chicken, International Conference on Biotechnology and Welfare In Animal Science with a Session on the 7th Poultry Days, Krakow

Kövesi B, Pelyhe Cs, Mézes M, Kovács B, Balogh K 2016, Short term Effects of T-2 toxin and deoxynivalenol on GPx4 gene expression in common carp (Cyprinus carpio L.) "FIBOK2016" Fiatal Biotechnológusok Országos Konferenciája, Program és összefoglalók p 55, Gödöllő

Kövesi B, Pelyhe Cs, Zándoki E, Mézes M, Balogh K, Kovács B 2016, Rövidtávú DON vagy T-2 toxin terhelés hatása az antioxidáns rendszer szabályozására ponty fajban (Cyprinus carpio) XL. Halászati Tudományos Tanácskozás, Szarvas

Pelyhe Cs, **Kövesi B,** Zándoki E, Mézes M, Balogh K, Kovács B 2016 DON és T-2 toxin együttes hatása az antioxidáns rendszer szabályozására ponty fajban (Cyprinus carpio) XL. Halászati Tudományos Tanácskozás, Szarvas

Kövesi B, Pelyhe Cs, Zándoki E, Mézes M, Balogh K, Kovács B 2016 Trichotecénvázas mikotoxinok együttes hatása az antioxidáns rendszer szabályozására rövidtávú toxinexpozíciót követően pontyban. Magyar Toxikológusok Társasága TOX'2016 Konferencia, Hajdúszoboszló, p. 68.

Pelyhe Cs, **Kövesi B**, Zándoki E, Mézes M, Kovács B, Balogh K 2017 Multi-mycotoxin effects on the regulation of the glutathione-redox system in broiler chicken; 39th Mycotoxin Workshop, Bydgoszcz

Pelyhe Cs, **Kövesi B**, Zándoki E, Mézes M, Balogh K, Kovács B 2017 Multi-mycotoxin effects on the regulation of the glutathione-redox system in common carp (Cyprinus Carpio L.); 39th Mycotoxin Workshop, Bydgoszcz

Ihos E, Pelyhe Cs, **Kövesi B**, Erdélyi M, Mézes M, Balogh K 2017 Effect of aflatoxins in farm animals; 6th Scientific day of animal breeding in Gödöllő, International Conference; Gödöllő

Pelyhe Cs, **Kövesi B**, Zándoki E, Mézes M, Kovács B, Balogh K 2017 Common effect of trichothecene mycotoxins on the glutathione-redox system and its regulation in broiler chicken; 6th Scientific day of animal breeding in Gödöllő, International Conference; Gödöllő

Pelyhe Cs, **Kövesi B**, Zándoki E, Kovács B, Mézes M, Balogh K 2017 Trichotecénvázas mikotoxin terhelés hatása pontyok (Cyprinus Carpio L.) glutation redox paramétereire és annak szabályozására; Magyar Szabadgyök-kutató Társaság IX. kongresszusa, Gödöllő, p. 29.

Kövesi B, 2017 Az aflatoxin és a deoxinivalenol pontyok antioxidáns rendszerének szabályozására gyakorolt hatásának vizsgálata; SZIE Kiváló Tehetségei Konferencia, Gödöllő; p. 69.

Ihos E, Pelyhe Cs, **Kövesi B**, Erdélyi M, Mézes M, Balogh K 2018 Effect of aflatoxins on the expressions of several genes of the antioxidant system in broiler chicken. LX. Georgikon Napok. Keszthely, p. 90 (ISBN 978-963-9639-91-1)

Kulcsár Sz, **Kövesi B**, Miklós M, Balogh K, Zándoki E 2018 Combined effect of some Fusarium mycotoxins on lipid peroxidation and glutathione redox systems in poultry. LX. Georgikon Napok. Keszthely, p. 90 (ISBN 978-963-9639-91-1)

Mézes M, Pelyhe Cs, **Kövesi B**, Zándoki E, Balogh K 2018 T-2 toxin és deoxinivalenol együttes hatása a lipidperoxidációra és a glutation-redox rendszerre, valamint annak szabályozására brojlercsirkében, Magyar Toxikológusok Társasága TOX'2018 Konferencia, Lillafüred, p. ?.

Kulcsár Sz, **Kövesi B**, Mézes M, Balogh K, Zándoki E, Ancsin Zs 2018 Egyes fusarium mikotoxinok együttes hatásának vizsgálata a baromfi lipidperoxidációs folyamataira és glutation rendszerére. Magyar Toxikológusok Társasága TOX'2018 Konferencia, Lillafüred,

Kulcsár Sz, **Kövesi B**, Mézes M, Balogh K, Zándoki E 2019 Effect of multi-mycotoxin exposure of Fusarium mycotoxins on glutathione redox system in poultry, 54th Croatian & 14th International Symposium on Agriculture: Book of Abstracts, pp. 38 ISSN: 2459-5551

Szabó-Rubina T, Kovács-Weber M, **Kövesi B**, Balogh K, Mézes M, Kovács B 2019 Mycotoxins induced DNA damage in common carp (Cyprinus carpio): investigations using LORDQ-PCR and DNA repair

gene expression analyses. 54th Croatian & 14th International Symposium on Agriculture: Book of Abstracts, pp. 175. ISSN: 2459-5551

Kövesi B, 2019 Az aflatoxin és deoxinivalenol együttes hatásának vizsgálata az antioxidáns rendszer szabályozására pontyok vese- és lép szöveteiben; SZIE Kiváló Tehetségei Konferencia, Gödöllő,

Kövesi B, Zándoki E, Mézes M, Balogh K 2019 Aflatoxin B1 és deoxinivalenol együttes hatásának vizsgálata az antioxidáns védőrendszer szabályozásra ponty fajban. Magyar Szabadgyök-kutató Társaság X. kongresszusa, Szeged, p. 32

Kulcsár Sz, **Kövesi B,** Mézes M, Balogh K, Zándoki E 2019 Egyes Fusarium mikotoxinok rövidtávú terhelésének hatása a baromfi glutation rendszerére és annak szabályozására. Magyar Szabadgyök-kutató Társaság X. kongresszusa, Szeged, p. 31

Balogh K, Zándoki E, Ancsin Zs, **Kövesi B,** Kulcsár Sz, Erdélyi M, Mézes M, Dobolyi Cs, Kukolya J 2019 Aflatoxinnal és szterigmatocisztinnel szennyezett takarmány hatása brojlercsirkék glutation redox és lipidperoxidációs paramétereire. Magyar Szabadgyök-kutató Társaság X. kongresszusa, Szeged, p. 28

Mézes M, Kulcsár Sz, **Kövesi B**, Zándoki E, Balogh K 2019 Fusarium mikotoxinok együttes hatásának vizsgálata rövidtávú terhelés során a baromfi glutation redox rendszerére és annak szabályozására, Magyar Toxikológusok Társasága TOX'2019 Konferencia, Szeged, p. 60.

Kövesi B, Kulcsár Sz, Zándoki E, Mézes M, Balogh K 2019 Ochratoxin A hatása a glutation-redox rendszerre valamint annak szabályozására brojlercsirkében, Magyar Toxikológusok Társasága TOX'2019 Konferencia, Szeged, p. 120

Kulcsár Sz, **Kövesi B**, Mézes M, Balogh K, Zándoki E 2019 Három együttesen előforduló Fusarium mikotoxin rövidtávú terhelésének hatása a brojlercsirke lipidperoxidációs folyamataira és glutation rendszerére, Magyar Toxikológusok Társasága TOX'2019 Konferencia, Szeged, p. 108.