# Interspecies variation in the hepatic biotransformation of zearalenone: Evidence for bio-inactivation of mycoestrogen zearalenone in sturgeon fish

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Received: November 2013 Accepted: August 2014

## **Abstract**

Zearalenone (ZEA) as mycoestrogen is found in human foods and animal feeds. Its potency depends on its biotransformation fate. biotransformation of ZEA in two species of sturgeon fish (Acipenser persicus and Huso huso) was investigated. ZEA was incubated with the hepatic microsomal and postmitochondrial sub-fractions in the presence of NADPH and the metabolites were determined by means of HPLC. Moreover, the rate of glucuronidation for ZEA and its metabolites were estimated in the presence of uridine diphosphateglucuronic acid. βzearalenol (β-ZOL) was found to be the major metabolite of ZEA by both subfractions. Enzymatic kinetics studies revealed that the maximum velocity  $(V_{max})$  in microsomal and post-mitochondrial fractions for β-ZOL production was found 5- and 7-folds in Huso huso and 8- and 12-folds in A. persicus higher than that for α-ZOL production, respectively. The H. huso hepatic post-mitochondrial fraction mainly glucurinated ZEA while in A. persicus, the metabolites and in particular β-ZOL were glucuronidated. Data suggest that the hepatic biotransformation of ZEA in studied sturgeons resulted in detoxification of ZEA as the main metabolite tends to be β-ZOL with weaker estrogenic property. Moreover, clear differences in glucuronidation profile are indicating interspecies variety in hepatic biotransformation of ZEA.

**Keywords**: *Acipenser persicus*; *Huso huso*; Hepatic biotransformation; Glucuronidation; Subcellular fractions; Zearalenone.

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# Introduction

Sturgeons as one of the most valuable aquatic resources are specifically found in large rivers, lakes, coastal waters and inner seas of the northern hemisphere. In addition of being a major source of income and employment, sturgeons' meat and caviar as unfertilized sturgeon roe are among the most valuable foods for human. Like other fish species, successful sturgeon farming also depends on the proper management and high quality water and feed. Several factors influence the feed quality including having complete nutrients and being free of contaminations. Mycotoxins are among the main feed contaminants, the substances which are produced by various fungi. Previous studies have shown that plant ingredients including vegetable oil, soybean meal, corn and wheat gluten make up the main parts of fish diet (Nizza and Piccolo, 2009). At the same been it has reported mycoestrogen zearalenone had been found at the highest level in the corn samples used for fish diet preparation. Recently ZEA was reported as the more prevalent mycotoxin with an average level of 67.9 µg kg<sup>-1</sup> fish feed (Pietsch et al., 2013).

ZEA, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)- $\beta$ -resorcylic acid lactone, is produced by *Fusarium* species including *F. culmorum* and *F. graminearum* (Hestbjerg *et al.*, 2002). These molds contaminate crops such as maize, barley and wheat (Yamashita *et al.*, 1995; Jimenez and Mateo, 1997).

The concentration of ZEA in feed materials can vary from few micrograms to 276 mg/kg up (Vrabcheva et al., 1996). Since ZEA binds to estrogen receptors (ERs) after ingestion and acts as an estrogenic compound, thus it is referred as a mycoestrogen (Malekinejad et 2005). Animals exposed to ZEA or **ZEA-contaminated** feed. show symptoms of hyperestrogenism. In this regards, previous data indicated that pigs are the most sensitive species to ZEA (Decasto et al., 1995; Yang et al., 1995). The estrogenisity of ZEA in rainbow trout has also been previously demonstrated by using the relative binding affinity method (Knudsen and Pottinger, 1999).

Previous investigations on effects of ZEA in fish have focused mainly on the reproduction of zebrafish (Johns et al., 2009). It is well documented that the estrogenic potency of ZEA could largely be affected by hepatic biotransformation and the conversion of ZEA into  $\alpha$ -zearalenol or  $\beta$ -zearalenol. We also have shown that there are species differences in hepatic biotransformation of ZEA in terms of different metabolite production and the rate of glucuronidation (Malekinejad et al., 2006). Sturgeons have a relatively long life span and depending on the species their sexual maturation starts at the age of six years onward, thus there are possibilities of consuming the ZEA contaminated diet. Although there is no direct report about the ZEAcontaminated **ZEA** sturgeon diet,

contamination has been previously reported in soils, drainage water, rivers and lakes of the United States and several other countries, ranged up to 220 ng/L (Maragos, 2012). In our previous study, we investigated the hepatic biotransformation of ZEA by subcellular fractions of rainbow trout and demonstrated that the hepatic resulted metabolism of **ZEA** detoxification lessening and the potency of estrogenic **ZEA** by converting it to weaker estrogenic metabolite of β-zearalenol (Malekinejad et al., 2012). As there is currently a lack knowledge about the hepatic biotransformation of ZEA in sturgeons, hence in this study the biotransformation of ZEA including both phases I and II reactions by subcellular fractions of H. huso and A. persicus were investigated.

#### Materials and methods

## Reagents

The test compounds ZEA,  $\alpha$ -zearalenol ( $\alpha$ -ZOL),  $\beta$ -zearalenol ( $\beta$ -ZOL), uridine diphosphate glucuronic acid (UDPGA), and nicotinamide dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade.

## Animals

Sixty sturgeons fish (average wt.  $200 \pm 5$  g) including *Huso huso* (n = 30) and *A. persicus* (n= 30) were obtained from the Artemia and Aquatic Animals Research Institute (Urmia, Iran) and

stocked in separate concrete ponds containing groundwater freshwater. The fish were cultured in a flow-through system with a flow rate of 50 L/min. Dissolved oxygen was maintained above 8 mg/L using constant aeration and fish were exposed to a natural photoperiod of approximately 12:12 L:D. The water temperature was 13.5  $\pm$ 1.0°C, and the pH was maintained between 7.3-7.5. The fish were fed on commercially standard and mycotoxin free formulated sturgeon diet (Esfahan Mokammel CO., Iran) for a period of three months.

Fish tissue collection and preparation of the liver subcellular fractions

The fish were kept in accordance with the guidelines of the Local Ethical Committee roles applying to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985 (NIH, 1985). For tissue collection, eight randomly collected male individuals (average wt.  $420 \pm 20$  g) were anesthetized by immersion in eugenol solution (20 mg/L) and immediately dissected to remove the liver. The collected liver samples were rinsed three times with chilled normal saline to get rid of the extra blood.

The sub-cellular fractions of the liver were prepared as described previously (Malekinejad *et al.*, 2005). Briefly, immediately after anesthesia, the liver specimens were collected and cut into small pieces. Two volumes of KCl (1.15%) and EDTA (0.1mM) solution were added to the tissue samples and

the mixtures were homogenized in a Potter-Elvehjem apparatus Teflon pestle (Krackeler Scientific Inc. Albany, NY, USA). The homogenates were centrifuged (Beckman Coulter Inc. USA) at 9,000 g for 30 min at 4  $^{\circ}$ C and an aliquot of the supernatant was collected as the post-mitochondrial fraction. The remaining sample was centrifuged at 100, 000 g for 90 min at 4 °C in order to obtain the microsomal fraction. After centrifugation, the supernatant was discarded and the pellet was carefully dissolved in the same volume of phosphate buffer (0.05 M; pH 7.4) with glycerol (20%), and then was homogenized with an ultraturrax homogenizer (T 25, IKA, Germany). The individual fractions extracted from the fish samples were pooled and stored at -70°C until use. Protein concentrations ofthe microsomal and the post-mitochondrial fractions were determined according to Lowry method (Lowry et al., 1951).

Incubation of ZEA with hepatic subcellular fractions (Phase I reactions)

To determine the biotransformation metabolites, various concentrations of ZEA (10, 25, 50, 100, 250, 500 μM, dissolved in methanol) were added to the reaction mixtures, containing either pooled liver microsomes (0.505 mg protein) or the post-mitochondrial fraction of the liver (2.008 mg protein) and supplemented with NADPH (0.5 mM) in a final volume of 250 μl phosphate buffer (50 mM, pH 7.4). The samples were incubated in a shaking

water bath (Memmert, Germany) at 20 °C for 30 min. The reaction was stopped by transferring the samples to an ice-cold environment followed by extraction with chloroform (1.25 mL). One milliliter of the organic phase was collected and evaporated to dryness under a gentle stream of N2. The residue was re-dissolved in the mobile phase and was subsequently analyzed by high performance liquid chromatography (Malekinejad et al., 2005).

Determination of the extent of glucuronidation

To evaluate the rate of glucuronidation, the post-mitochondrial fraction was dialyzed overnight using cellulose membranes (width 25 mm, diameter 16 mm; retaining proteins with a MW>12 KDa and hence all enzymes) with a dialysis buffer containing 1.15% (w/v) KCl and 0.1 mM EDTA, which was refreshed twice. Subsequently, increasing concentrations of ZEA were added to the dialyzed fraction (2.008 mg protein) dissolved in a phosphate (50)mM, pН 7.4) supplemented with 0.5 mM NADPH (pre-dissolved in phosphate buffer) in the absence or presence of 10 mM UDPGA, and incubated in shaking water bath for 30 min at 20°C. The reaction was stopped by placing the samples on ice and adding 1.25 ml icecold chloroform. After vigorous mixing and centrifugation at 3000 g for 5 minutes at 4°C, one ml of the organic phase was collected and evaporated to dryness under a stream of nitrogen. The residue was subsequently analyzed by HPLC. The rate of glucuronidation (%) was calculated using the following equation:

% of glucuronidation =  $100 - (100 \times 100)$  the amount of produced metabolites in the presence of UDPGA / the amount of produced metabolite in the absence of UDPGA)

Determination and confirmation of ZEA metabolites using HPLC method ZEA and its metabolites determined high-performance using liquid chromatography (HPLC), to previously described according method (Malekinejad et al., 2005). Shortly, the chromatographic system consisted of an auto sampler (Autosampler Triathlon type 900. Germany) and dual pumps (Wellchrom HPLC pump, K-1001, **KNAUER** Germany). Twenty microlitres of the extracted sample was injected into a C18 (150×4.60 LUNA 5μ Phenomenex) column. The mobile phase consisted of a mixture of methanol- water (70:30, v/v) eluted at a flow-rate of 0.8 mL/min. ZEA and its metabolites were detected by means of a fluorescence detector (RF-10AXL KNAUER. Germany). set excitation and emission wavelength of 236 and 418 nm, respectively. ZEA and its metabolites were quantified by measuring peak areas and comparing them to the relevant calibration curves. To obtain calibration curve for each single compound, we subjected a

concentration range from zero to 100  $\mu M$  ( $r^2$ = 0.9992 for ZEA,  $r^2$ = 0.9975 for  $\alpha$ -ZOL and  $r^2$ =0.9985 for  $\beta$ -ZOL).

Statistical analysis

All results are presented as means ± SD. For kinetic studies the Michaelis-Menton  $(K_m)$ and constant the maximum velocity  $(V_{max})$ were obtained. Differences between the amounts of the individual products were analyzed with a two-way ANOVA followed by a Bonferroni test, using Graph Pad Prism 4.00, Graph Pad Software. p < 0.05was considered significant.

#### Results

ZEA was bio-inactivated by both sturgeons' hepatic biotransformation Incubation of ZEA with the hepatic subcellular fractions of sturgeons, followed by chloroform extraction and HPLC analyses, revealed that although both known metabolites of ZEA including  $\alpha$ - and  $\beta$ -ZOL are produced by either studied fractions, however β-ZOL found to be the dominant metabolite. Fig. 1 shows both standard and sample chromatograms with identical retention time for individual compounds. The β-ZOL production by Huso huso hepatic microsomes was found 3 fold higher than that of A. persicus. We did not find significant differences (p>0.05)the level between of produced metabolites by two post-mitochondrial fractions of the two different species of studied sturgeons.

Comparison of the biotransformation capacity of the studied fractions showed that the microsomal fraction produced significantly (p<0.05) more quantities of both metabolites than those produced by post-mitochondrial fraction in both examined fishes (Fig. 2).

Enzymatic kinetics studies revealed that the maximum velocity  $(V_{max})$  in microsomal and post-mitochondrial fractions of H. huso for β-ZOL production was found approximately 5and 7-fold higher than that for  $\alpha$ -ZOL production, respectively. value for the  $\alpha$ -ZOL production by hepatic microsomal fraction of H. huso was 1.7-fold higher than that for the β-ZOL production, while it was found in contrary in the post-mitochondrial fraction as the Km value for β-ZOL production was approximately 3-fold than that for the  $\alpha$ -ZOL production (Table 1). At the same time, the  $V_{max}$  values for  $\beta$ -ZOL production by the hepatic microsomal and postmitochondrial subfractions of persicus was estimated 8- and 12-folds higher than that for  $\alpha$ -ZOL production. The highest Km value was found for β-ZOL production bv the postmitochondrial fraction in A. persicus.

Glucuronidation of ZEA and its hydroxylated metabolites

The rate of glucuronidation of ZEA and its hydroxylated metabolites by the post-mitochondrial fraction of

sturgeons was determined. The first finding of the conjugation revealed that there is a clear difference between two sturgeons in terms of glucuronidation profile as H. huso hepatic post-mitochondrial fraction mainly glucurinated ZEA while in A. persicus, the metabolites particular β-ZOL were glucuronidated. We found that *Huso huso* conjugated  $\alpha$ -ZOL significantly (p<0.05) more than β-ZOL, while A. persicus postmitochondrial sub-fraction mainly β-ZOL. glucuronidated Our results showed level reverse of glucuronidation the high concentration of ZEA. the percentage of glucuronidation, indicating a saturable condition of glucuronidase, which is responsible for glucuronidation of ZEA and its reduced metabolites (Table 2).

### Discussion

The hepatic biotransformation of ZEA types of in two sturgeon investigated. The results showed that, although there are some similarities in the production of  $\beta$ -ZOL as the dominant metabolite by hepatic subfractions of both either sturgeons, but there are also substantial differences in the glucuronidation of ZEA and its metabolites post-mitochondrial by fractions.

Table 1: Kinetics of ZEA biotransformation by the sturgeon's hepatic subcellular fractions.

Metabolite	Microsomes	Post-mitochondrial
Huso huso		
(α-ZOL)		
V <sub>max</sub> (pMol/min/mg)	$19.5 \pm 2.5$	$2.9 \pm 0.09*$
$K_{\rm m}(\mu { m Mol}/{ m L})$	$91.2 \pm 34.6$	$16.9 \pm 2.5*$
Γ-	0.68	0.88
β-ZOL		
V <sub>max</sub> (pMol/min/mg)	$98.8 \pm 6.5$	$20.8 \pm 1.04*$
$K_{\rm m}  (\mu \dot{M}ol/L)$	$52.8 \pm 11.9$	$55.25 \pm 9.4$
$r^2$	0.85	0.91
A. persicus		
A. persicus (α-ZOL)		
V <sub>max</sub> (pMol/min/mg)	$3.2 \pm 0.08$	$1.7 \pm 0.06$ *
$K_{\rm m}(\mu { m Mol/L})$	$13.01 \pm 1.7$	$28.5 \pm 4.4*$
$r^2$	0.90	0.89
0.701		
β-ZOL	25.4 . 1.2	21.2 - 1.02*
V <sub>max</sub> (pMol/min/mg)	$25.4 \pm 1.2$	$21.3 \pm 1.02*$
$K_{\rm m} (\mu { m Mol}/{ m L})$	$17.3 \pm 3.8$ 0.76	72.3 ± 10.9 0.95
1	0.70	0.33

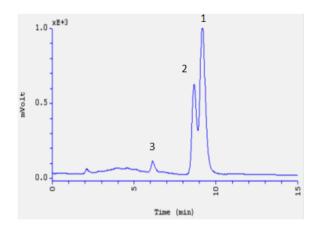
Table 2: Percentage of glucuronidation of ZEA and its metabolites by the hepatic postitochondrial fraction of rainbow trout.

ZEA (µM)	ZEA	α-ZOL	β-ZOL
Huso huso			
10	$71.7 \pm 9.1$	$33.1 \pm 2.8$	$16.3 \pm 1.2$
25	$64.9 \pm 8.0$	$29.0 \pm 0.5$	$9.6 \pm 1.8$
50	$45.7 \pm 13.9$	$27.5 \pm 1.3$	$6.4 \pm 0.3$
125	$40.2 \pm 2.7$	$26.1 \pm 2.2$	$5.5 \pm 3.3$
250	$29.5 \pm 12.4$	$11.9 \pm 1.1$	$4.4 \pm 0.6$
500	$19.7 \pm 4.6$	$4.7 \pm 2.3$	$3.2 \pm 1.1$
A. persicus			
10	$23.6 \pm 1.1$	100	100
25	$24.0 \pm 2.3$	100	100
50	$16.7 \pm 2.9$	$31.2 \pm 0.7$	100
125	$13.2 \pm 1.3$	$28.7 \pm 0.4$	$44.0 \pm 0.3$
250	$7.3 \pm 0.5$	$23.3 \pm 3.1$	$24.5 \pm 0.5$
500	$5.7 \pm 0.6$	$19.2 \pm 0.6$	$12.3 \pm 0.9$

Very first finding of the current study was that the hepatic biotransformation of ZEA resulted in detoxification as  $\beta$ -ZOL with rather weaker estrogenic activity was found as the main metabolite in both sturgeons. There are

numerous studies indicating that  $\beta$ -ZOL in comparison to  $\alpha$ -ZOL and the original compound (ZEA) is a weaker mycoestrogen (Le Guevel *et al.*, 2001; Malekinejad *et al.*, 2005; Filannino *et al.*, 2011).

A)



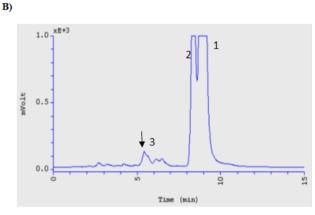


Figure 1: Standard (A) and sample chromate grams (B) with identical retention time for individual compounds; 1: Zearalenone, 2: α-Zearalenol and 3: β-Zearalenol.

Cosnefroy and co-workers recently demonstrated the same order estrogenic potency for ZEA and its metabolites reduced by using zebrafish specific in vitro stable reporter gene assay (Cosnefroy et al., 2012). Comparing two examined sturgeons indicates that there are clear differences between the amounts of produced metabolites by the hepatic sub-fractions of two sturgeon species, suggesting interspecies variety in the **ZEA** biotransformation. hepatic Although there is no direct report about the ZEA biotransformation in sturgeons, however our previous studies in mammalians showed considerable interspecies differences between various ruminants including cattle and sheep (Malekinejad *et al.*, 2006).

Enzymatic analyses of this study clarified that  $V_{max}$  for production of both metabolites in microsomal fraction was higher than that in postmitochondrial fraction in both tested sturgeons, indicating dense localization of enzymes in microsomal fraction.

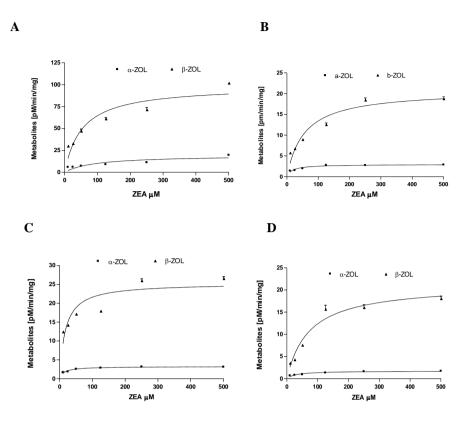


Figure 2: Biotransformation of ZEA by A) huso huso hepatic microsomes, B) huso huso hepatic post-mitochondrial fraction, C) A. persicus hepatic microsomes and D) A. persicus post-mitochondrial fraction.

This finding is in a good accordance with previous works (Malekinejad et al., 2005; Malekinejad et al., 2006; malekinejad et al., 2012). Our data suggest a higher efficiency (V<sub>max</sub>/K<sub>m</sub> ratio) of the microsomal enzymes than post-mitochondrial enzymes in examined sturgeon's liver and this finding is not consistent with previous finding in the rainbow trout (Malekinejad et al., 2012) suggesting various affinity of enzymes toward the ZEA between different species of fish. Additionally, under the assumption that  $3\beta$ -HSD converts ZEA into  $\beta$ -ZOL, the obtained enzyme kinetics indicate a higher efficiency of  $3\beta$ -HSD than  $3\alpha$ -HSD in examined sturgeon's liver. Our indirectly data also confirm

expression and localization of both isoenzymes albeit with differences in the liver of examined sturgeons.

We also investigated the glucuronidation rate of ZEA and its reduced metabolites by the hepatic post-mitochondrial fraction of sturgeons. It is known that the rate of glucuronidation mainly depends on the capacity of UDPGTs and availability of UDPGA (Belanger et al., 1990; Tukey and Strassburg, 2000). The estimated percentage of the conjugation rate for all three compounds indicates that there are profound differences between two examined sturgeons in terms glucuronidation profile of ZEA and its metabolites. The main finding in this regard is indicating that the H. huso

hepatic post-mitochondrial fraction mainly glucuronidated ZEA, while ZEA reduced metabolites were largely glucuronidated Α. persicus, by indicating another difference between two sturgeons in the biotransformation of ZEA. There is molecular evidence indicating the presence of multiple UGT genes in zebra fish, suggesting the fact that the different glucuronidation profile of ZEA and its metabolites in our study may be related to the of UDPpresence distinct glucuronosyltransferases in two sturgeons (George examined and Taylor, 2002). Nevertheless, clarifying the presence and function of UGT enzyme(s) in sturgeons is required.

The data described in the present study is the first report showing that ZEA is converted mainly into  $\beta$ –ZOL which could account for detoxification method. Moreover, different glucuronidation profile of ZEA and its metabolites in two examined sturgeons confirmed the interspecies variety of the ZEA hepatic biotransformation.

# Acknowledgement

This study was financially supported by the Artemia and Aquatic Animals Research Institute under the grant number of 004/A/86.

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