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Effects of the pesticide methoxychlor on gene expression in the liver and testes of the male largemouth bass (*Micropterus salmoides*)

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Abstract

The organochlorine pesticide methoxychlor (MXC) is an environmental estrogen known to stimulate the expression of the egg-yolk protein, vitellogenin (Vtg) in fish species. To begin to understand the underlying mechanisms for how MXC exerts its deleterious effects on the endocrine system, male largemouth bass (*Micropterus salmoides*) were treated with 2.5, 10, or 25 mg/kg MXC and compared to fish pair-treated with 1 mg/kg 17 β -estradiol (E₂), and vehicle control. Fish were sacrificed 24, 48, or 72 h following treatment. The liver and testes were then assayed for changes in expression of the three bass estrogen receptors (ERs α , β a, and β b) in tissues, as well as Vtg and cytochrome P450 (CYP) 3A isoform 68 in the liver and steroidogenic acute regulatory protein (StAR) in the testes. In the liver, significant increases in gene expression were seen for each of the genes measured by 24 h and each returned to the level of the vehicle by 72 h. Total testosterone 6 β -hydroxylase activity, reflective of CYP3A activity, was also increased by 24 h for all of the exposures. In the testes, ER α was unaffected by any treatment, ER β a was upregulated only by MXC, peaking at 24 h for the 2.5 and 10 mg/kg MXC and at 48 h for the 25 mg/kg MXC treatment. By 72 h, the MXC effects had disappeared, while E₂ significantly decreased the expression of ER β a mRNA. ER β b expression in the testes was stimulated by all concentrations of MXC by 24 h and the effect remained up to 72 h, whereas E₂ had no effect. Finally, StAR expression was found to also be decreased by E₂ and all MXC treatments. However, the effect on StAR expression by E₂ occurred within 24 h, while the effect by all concentrations of MXC was not seen until 72 h after treatment. The stimulatory effects of E₂ and 25 mg/kg MXC on the expression of the ERs in the liver were opposite to the responses seen in the testes, suggesting an inverted relationship between these two tissue types. These results provide a possible mechanism showing that alterations in reproductive signaling in male fish by xenoestrogens not only increase Vtg expression in the liver, but may also decrease reproductive success by muting some of the estrogen signals required for sperm production.

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Keywords

methoxychlor; estradiol; estrogen receptors; largemouth bass; gene expression

Introduction

The advent of pesticide use in agriculture has been, and continues to be, one of the most important developments for the high level of food production required for our modern society. Unfortunately, many of these chemicals have had unintended effects on wildlife species in the environment. Many of these effects are due to the pesticides interacting with the estrogen receptors (ERs) and other regulators of reproduction. These xenoestrogens are able to stimulate transcriptional activation of the ERs, either through direct interaction with them, or through an indirect mechanism. One of these agents is the pesticide methoxychlor (MXC).

MXC was introduced following the banning of DDT in the United States in 1972. It is structurally similar to DDT (Figure 1), but is less environmentally persistent, having an approximate environmental half-life of 120 days (versus 2-15 years reported for DDT (Augustijn-Beckers et al., 1994; US E.P.A., 1989)), depending on the amount of aerobic activity in the soil (Fogel et al., 1982; Wauchope et al., 1992). According to the National Center for Food and Agricultural Policy National Summary (1997), almost 78,000 pounds of MXC were applied principally to fruit crops in the United States in 1997, with about 86% being applied to apple trees. It is highly toxic to fish with a 96-hour LC₅₀ value for the technical grade (90% pure) of less than 20 µg/l for largemouth bass and between 20 and 65 µg/l for fathead minnows (for comparison) (Johnson and Finley, 1980). It is also more highly metabolized *in vivo* than DDT (Kapoor et al., 1970).

MXC has been classified as a pro-estrogen, requiring activation through demethylation of the methoxy groups. This has been shown to be catalyzed by cytochrome P450 (CYP) enzymes 1A in rats (Dehal and Kupfer, 1994), 1 and 3A in channel catfish (Stuchal et al., 2006), or 1A2 and 2C19 in humans (Stresser and Kupfer, 1998). The estrogenic metabolites are the mono- and bis-demethylmethoxychlor compounds (OH-MXC and HPTE, respectively) produced by the action of the CYP enzymes.

In male fish, MXC has been shown to stimulate the gene for the estrogen-responsive egg-yolk precursor vitellogenin (Vtg) in males. These reports include the zebrafish (Ortiz-Zarragoita and Cajaraville, 2005), channel catfish (Nimrod and Benson, 1997), carp (Rankouhi et al., 2004) and sheepshead minnow (Hemmer et al., 2001). Using gene arrays, Larkin et al., (2003) reported changes in gene expression due to MXC exposure in sheepshead minnow (SHM). The genes that were up regulated on the arrays from MXC-treated fish resembled those up regulated by estradiol (E₂)-treated fish and included Vtgs 1 and 2, as well as ERα. In addition, MXC treatment also up regulates one of the zona radiata proteins in sheepshead minnows as has been shown by SELDI-TOF-MS (surface-enhanced laser desorption ionization-time of flight mass spectroscopy) (Walker et al., 2007).

Much of the other work in non-mammals has focused on the biotransformation of MXC and the downstream effects on steroid biosynthesis. Either or both of these pathways can have downstream endocrine disrupting effects by increasing or decreasing the levels of endogenous steroid hormones. One report by Ohyama et al. (2004) compared the metabolism of MXC in rodents, bird, and fish liver slices. They found some differences in the metabolites produced. Each species produced the OH-MXC metabolite, while the HPTE metabolite was only detected in rodents and fish. Using channel catfish, Stuchal et al. (2006)

found that pre-treatment with MXC led to decreased metabolite formation by intestinal microsomes, while there was no significant effect on the biotransformation by liver microsomes.

Far more work has been done looking at the effects in mammals. However, like in non-mammals, much of the *in vivo* work has gone into examining the phenotypic effects of MXC treatment, rather than the effects on gene expression. *In vivo* studies have shown that prepubertal male rats receiving MXC in the diet had greater mammary gland development than pair-treated controls (You et al., 2002). Uterine growth weight was increased, as well as uterine expression of vascular endothelial growth factor-2 and angiotensin-1, while the pituitary size was decreased (Goldman et al., 2004). Several other reproductive-related effects have also been documented in rats like precocious vaginal opening (Eroschenko and Cooke, 1990) and in mice, difficulties with initiating and maintaining pregnancy (Swartz and Eroschenko, 1998). Each of these effects is associated with ER activity. MXC was shown to bind to both the constitutive androstane receptor (CAR) and the pregnane $\times$ receptor (PXR) and induce CYP2B and CYP3A mRNA and protein in rats (Blizard et al., 2001, Mikamo et al., 2003). Induction of these two CYPs may affect steroid hormone metabolism.

In order to gain some insight into the phenotypic effects seen with MXC exposure in the largemouth bass (LMB), it is important to understand how it may alter gene expression. MXC is known to stimulate gene expression through the ER in both fish (Nimrod and Benson 1997) and mammals (Miller et al., 2006). We were interested in comparing ER stimulation by MXC and E_2 in regard to their effects on the expression of the ERs themselves as well as the principle estrogen biomarker, Vtg 1, in male LMB. An additional gene of interest was CYP3A isoform 68, since CYP3A is implicated in the biotransformation of MXC to its more estrogenic metabolites. The aims of this study were to determine if MXC affects expression of the three ERs in either the liver or testes in male LMB, the expression of Vtg 1 and CYP3A68 (a recently cloned isoform of CYP3A (Barber et al., 2007)) in the liver, and the steroidogenic acute regulatory protein (StAR) in the testes. Each of these genes represents an important facet of regulation of the reproductive process, and altered expression may lead to diminished reproductive success in male LMB.

Materials and Methods

Animals

Adult LMB were purchased from American Sport Fish Hatchery (Montgomery, AL). Fish were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Adult male LMB were separated from females by massaging the ventral surface of the fish to cause visualization of semen from the genital opening. This was necessary because male and female bass are not sexually dimorphic. Following sexing, the males were placed into a separate holding tank for 24 h until treatment. Prior to injections, fish were first anaesthetized in water containing 50-100 ppm of the fish anesthetic agent tricaine methane sulfonate, MS-222 (Sigma, St. Louis, MO). All treatment solutions of E_2 (Sigma) and MXC (Sigma) were made up to deliver the treatment via injection of 1 μ L per gram bodyweight. Following injection, the fish were dipped into a saltwater bath to kill any pathogens introduced by handling and to stimulate mucus layer secretion on the skin.

Exposure of adult male LMB to MXC and 17 β -estradiol (E_2)

A pilot study to find a vehicle suitable to administer MXC via injection was performed to find one that could be safely used in bass. Initially DMSO was tested, resulting in high mortality of LMB, probably due to rapid release of MXC into the system (unpublished

observation). Since sesame oil was effectively used in mice to deliver MXC (Eroschenko et al., 1996; Borgeest et al., 2004), it was used for LMB. A stock solution of MXC of 400 mg/mL was made in acetone from which appropriate dilutions were then made in acetone and mixed with sesame oil (purchased from a local grocery store) to give final concentrations of 2.5, 10, 25 mg/kg MXC in a vehicle containing 6.25% acetone and 93.75% sesame oil. There were no visible signs of distress in treated LMB for the duration of the pilot study.

After finding no visual signs of acute toxicity in the pilot fish, a total of 81 male LMB were simultaneously treated with either vehicle (n=9), 1 mg/kg E₂ (n=9), 2.5 mg/kg MXC (n=18), 10 mg/kg MXC (n=18), or 25 mg/kg MXC (n=18). After the injection, the fish were placed in tanks sorted by treatment. The fish were all injected on the same day (time=0) and then put into tanks for 24, 48, or 72 h. One third from each treatment group was collected at each of the time points. The fish were anaesthetized and killed by blunt head trauma and blood was collected, along with liver and testes. The tissues were chopped into pieces, frozen in liquid nitrogen, and stored at -80°C until analyzed or extracted for RNA.

Extraction of Methoxychlor and Metabolites from Liver

Standards for the MXC metabolites, OH-MXC and HPTE, were obtained by boron tribromide demethylation of MXC, as described previously (Stuchal et al., 2006) for validating the extraction procedure. Portions of liver, approximately 1 g, were accurately weighed and homogenized in 3 mL of 10 mM ammonium acetate pH 4.6 buffer. Acetonitrile:ethanol (2:1 by volume), 2 mL, was added and the mixture was vortex-mixed then sonicated for 10 minutes and vortex-mixed again. The mixture was centrifuged for 20 minutes at approximately 5,000 X g, the supernatant was transferred to clean vials, and the pellet re-extracted with 2 mL acetonitrile:ethanol. The combined organic extracts were evaporated to dryness in a Speed-Vac (Savant, Holbrook, NY). The residue was dissolved in 5 mL of 20% acetonitrile in 10 mM ammonium acetate pH 4.6 buffer and applied to a C₁₈ Sep-pak (Waters) that had been preconditioned with methanol, water and 20% acetonitrile in 10 mM ammonium acetate pH 4.6 buffer. The Sep-pak was washed with 5 mL 20% acetonitrile: 80% 10 mM ammonium acetate pH 4.6 buffer then with 5 mL 90% acetonitrile: 10% 10 mM ammonium acetate pH 4.6 buffer and finally with 5 mL 100% acetonitrile. The fraction containing the analytes (90% acetonitrile) was evaporated to dryness, taken up in 300 µL of mobile phase, filtered through a 0.45 µm nylon filter and analyzed by reverse-phase HPLC. To test the efficiency of extraction, portions of liver from untreated fish were spiked with known amounts of MXC, OH-MXC and HPTE and treated as for samples of dosed liver.

HPLC Analysis

HPLC analysis of MXC and its demethylated metabolites, OH-MXC and HPTE was carried out on a Beckman Gold System equipped with UV (Beckman Coulter, Fullerton, CA) and radioactivity (IN/US systems β-RAM, Tampa, FL) detectors. Reverse phase (C₁₈) separation was carried out at 40°C, using a 25cm × 4.6 mm Discovery[®] column with a particle size of 5µm fitted with a 2 cm × 4.6 mm guard column (Supelco, Bellefonte, PA). A gradient elution program was operated at 1 mL/min flow rate, starting with a solution of 30% acetonitrile in 10 mM ammonium acetate/acetic acid buffer (pH 4.6) held for 0.5 minutes then gradually increasing to 90% acetonitrile for 20 minutes and then to 100% acetonitrile for another 5 minutes. Compounds were detected at 245 nm and identified by comparison of retention times with those of authentic standards. Calibration curves were prepared from solutions of MXC, OH-MXC and HPTE of known concentration over the range 40 to 2500 pmol per 200 µL injection (Nyagode, 2007).

Direct Gene Cloning

A fragment of LMB 18S rRNA was cloned by amplifying a segment from LMB liver cDNA using the Human 18S rRNA primers (Applied Biosystems Cat #4310893E). The purified cDNA was cloned into pGEM-T Easy and sequenced, allowing LMB-specific primers to be designed. For Vtg 1, a fragment was re-cloned corresponding to position 635 to 1133 of the Vtg 1 sequence, (Genbank Accession AF169287) using the forward primer GGATACAGTCCCTGCCATTG and reverse primer CATTCTGGGTTCCCTTCTTTTGC which flank the previously published primer set for real time PCR from Sabo-Attwood et al. (2004). For each of these gene products, the PCR product was gel purified, cloned into pGEM-T Easy (Promega), and sequenced to ensure that the expected sequences were obtained.

Tissue RNA Extraction and cDNA Synthesis

Total RNA was isolated using TriZOL (Gibco) as directed by the manufacturer using ~100 mg of tissue per mL of TriZOL. The resulting RNA pellet was solubilized in RNA Secure reagent (Ambion) and quantified by measuring the absorbance at 260 nm and the relative purity was evaluated by computing the ratio of A_{260} to A_{280} where a ratio of 1.8 to 2.0 was considered highly pure. Ten micrograms of total RNA were then treated with DNase I using DNA-free (Ambion) as described by the manufacturer. Random samples were checked by gel electrophoresis in MOPS-formaldehyde gels to evaluate RNA integrity.

Total RNA was reverse transcribed using Stratascript (Stratagene). A total of 2 µg of DNase I treated RNA was combined with 1.8 µL of random hexamer primers (100 ng/µL) and water for a final volume of 24.6 µL. This mixture was incubated at 65°C for five minutes and cooled slowly to room temperature for 10 minutes to anneal the primers to RNA. After cooling, 5.4 µL of reverse transcription master mix (3 µL 10× Stratascript buffer (Stratagene), 0.6 µL RNase Block (40 U/µL, Stratagene), 1.2 µL dNTP (100mM, Invitrogen), 0.6 µL Stratascript Reverse Transcriptase (50 U/µL, Stratagene) were added to each reaction. The reaction mixture was mixed and incubated at 42°C for one hour and then the enzyme was inactivated at 90°C for five minutes.

Quantitative Real Time PCR

To determine the number of copies for each gene of interest present in the samples, 1 µL of cDNA (synthesized above) was used in a 25 µL reaction containing 1× SYBR green master mix (Applied Biosystems), forward and reverse primers (see Table 1 for sequences and concentrations), 0.01 µM fluorescein (Biorad) and nuclease-free water. Each hepatic or testicular cDNA was assayed on a single reaction plate to reduce plate to plate variability, and then each plate was duplicated to get replicate values per sample. In addition to the samples, each plate contained a standard curve for the gene of interest made from serial dilutions of the vector construct containing the gene, a standard reference pool, to correct for any differences between duplicate plates, and a no template control.

Linearity of the standard curve was determined from the slope of a plot of threshold cycle (C_t) versus the concentration. A slope of 3.322 represents complete doubling of the cDNA in each cycle ($2^{3.322}=10$). For genes of interest, standard curves were diluted in water using serial 10-fold dilutions ranging from 10^{-10} to 10^{-6} copies and for the housekeeping gene, 18S rRNA, the curve ranged from 10^{-5} to 10^{-8} copies per microliter. For the 18S rRNA, the standard curve was linear only through a concentration of 10^{-8} copies per microliter. The 18S rRNA was used to normalize the data as described in the statistics section below. The standard curves were made from stock solutions of plasmids containing a portion of the particular gene and were linear (correlation coefficient > 0.98). Calculated PCR efficiencies ranged from 99.6% to 105.3%. The gene segments used for the standard curve for 18S rRNA, Vtg

1, CYP3A68 (Barber et al., 2007), and StAR (Kocerha, 2005) were cloned into the pGEM-T Easy vector (Promega). The curves for the three ERs were made from dilutions of pCMV4 expression vectors (Stratagene) (Sabo-Attwood et al., 2007).

Subcellular Fractionation

Subcellular fractionation of LMB liver was done as previously described (James and Little, 1983). Frozen liver was thawed on ice and combined with 4 volumes of ice-cold liver homogenization buffer (150 mM potassium chloride, 50 mM potassium phosphate (pH 7.4), and 0.2 mM PMSF) and homogenized. Homogenates were first centrifuged at 13,000 X g for 20 min. The supernatant was centrifuged again for 45 min at 140,000 X g. The resulting microsomal pellet was washed in homogenization buffer and then centrifuged at 140,000 X g for 30 min. The pellet containing washed microsomes was resuspended in a volume equal to the original wet weight of the liver in resuspension buffer (250 mM sucrose, 10 mM HEPES (pH 7.4), 5% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.1mM PMSF). The resuspended microsomes were stored under nitrogen at -80°C until use in enzyme assays. Prior to assaying for activity, the microsomal protein concentration was measured using Lowry assay (Lowry et al., 1951) using bovine serum albumin as the standard.

Measurement of Testosterone 6 β -hydroxylase Activity

Testosterone hydroxylation was measured using the method of Lou et al. (2002). Assay tubes contained 1 mg of LMB liver microsomes, 0.1 M HEPES pH 7.6, 2 mM MgCl₂, 2 mM NADPH and 0.2 mM [4-¹⁴C]-testosterone (0.13 μ Ci per assay – Dupont NEN TM, Boston MA) in a total volume of 0.5 mL. Tubes were incubated for 10 minutes at 35°C, and products were extracted and quantified as described (Lou et al., 2002).

Statistical Analysis

For PCR data analysis in these experiments, the log₁₀ of the starting quantity of message was obtained from the standard curves. The i-Cycler software gives the standard curve in the form of $y=mx+b$. This equation can be re-organized to $x=(y-b)/m$, where x is the log₁₀ of the number of copies of the message in the particular sample, y is the Ct value of the particular sample, b is the y-intercept of the line, and m is the slope. A slope and y-intercept were obtained for each gene and the equation $\log_{10}(\text{copy number}) = [\text{Ct}-(\text{y-intercept})]/\text{slope}$ was used to calculate the log₁₀ of the copy number based on a Ct value for each sample. The mean Ct value for each sample run in duplicate was computed and normalized to the log₁₀ (copy number) of the 18S. Then the log₁₀ (copy number) was adjusted to 1 μ g RNA per reaction. To test the effects of MXC, the model included the main effects of treatment, time, and the interaction of these effects, treatment $\times$ time. When the treatment effect was found to be significant ($p < 0.05$), the data were then sorted by time and compared by ANOVA using treatment as the main effect. When a significant effect was found by ANOVA, the treatments were compared using Duncan's multiple range test to determine specific differences among the treatment groups. The fold-changes of the treatment groups compared to vehicle were computed by taking the differences of the log₁₀ (copy number) and analyzed using ANOVA. All statistical analyses were performed using the Statistical Analysis Software (SAS) package. Data presented are the mean log₁₀ (copy number) of the gene expression $\pm$ SEM.

Results

Changes in Gene Expression in the Liver by Treatment is Dependent on Time

It was of interest to determine how the expression of these genes would vary over the 72 h time course. The results of these analyses are presented on Figures 2 A-E. In general,

increases in gene expression compared to the vehicle alone were seen by 24 h post-treatment and they returned to the level of vehicle by 72 h. We are unsure as to why treatment with the vehicle alone tended to increase gene expression of our target genes. Possibly this was due to impurities in the sesame oil. However, in every case, the treatment effects were compared to the effects of vehicle alone and were statistically significant as discussed below.

ER α expression (Figure 2A) was most increased at 24 h post-treatment by E₂ (39.8-fold), followed by treatment with 25 mg/kg MXC (12.6-fold). After 48 h, the magnitude of stimulation for the E₂ treatment group had begun to decrease, while that for 25 mg/kg MXC continued to increase to its maximum level (34.7-fold). Fish treated with 10 mg/kg MXC were found to have a maximal response by 48 h, at which time ER α levels were approximately equal in magnitude to the stimulation by E₂ (6.0-fold). By 72 h, all treatment groups were no longer different from vehicle.

ER β a (Figure 2B), ER β b (Figure 2C), Vtg 1 (Figure 2D) and CYP3A68 (Figure 2E) showed similar expression patterns. For all of these genes, the highest expression over control was observed at 24 h for all of the treatments. For ER β a, E₂, 2.5 mg/kg, and 10 mg/kg MXC stimulated expression by an average of 4.4-fold. The 25 mg/kg MXC group was significantly higher than the other treatment groups, reaching a level of 15.5-fold induction by 24 h. By 48 h, the responses seen across all treatment groups were no longer different from vehicle control although the level of expression was still higher for 25 mg/kg MXC than the other treatment groups. For ER β b the responses for E₂, the 2.5 mg/kg and 10 mg/kg MXC were about 9.5-fold greater than vehicle at 24 h. Treatment with 25 mg/kg MXC increased expression by 22.4-fold. By 72 h, all treatments except the 2.5 mg/kg MXC were indistinguishable from vehicle with the 2.5 mg/kg MXC treatment significantly down-regulating ER β b (to about 20% of vehicle).

Vtg 1 was the most highly up regulated mRNA. E₂ induced the greatest increase (6,166-fold), followed by 25 mg/kg MXC (776-fold) and then by the 2.5 mg/kg and 10 mg/kg MXC (about 158-fold). By 48 h, the only treatment still causing a significant increase was E₂ (21.2-fold). At the 72 h time point, the effects of the treatments were no longer different from vehicle, although E₂ still caused an effect that was significantly greater than the MXC treatment groups.

CYP3A68 was significantly up regulated only at 24 h by all treatments with 25 mg/kg MXC giving the greatest increase (20.4-fold), followed by E₂ which was no different from the 2.5 mg/kg and 10 mg/kg MXC groups (about 6.7-fold compared to the vehicle control).

Hepatic Testosterone 6 β -hydroxylase Activity is Increased by Treatment

In the liver, testosterone 6 β -hydroxylase activity was increased by each of the treatments by 24 h following treatment (Figure 3). This suggests that the increase in CYP3A68 mRNA is translated into protein and into increased activity for all treatment groups. Compared to vehicle, 1 mg/kg E₂ produced a 2.3 fold induction in activity, which was similar to the 2.4 fold increase induced by treatment with 10 mg/kg MXC. At 25 mg/kg, MXC caused an increase of 2.7 fold in activity, while the greatest increase was seen with the 2.5 mg/kg MXC treatment which induced activity by 3.3 fold. The effects of all treatments on mRNA induction compared to vehicle were gone by 48 h after treatment.

Changes in Gene Expression in the Testes by Treatment is Dependent on Time

Like the changes seen in the liver, changes in gene expression in the testes are time-dependent following a single IP injection of each treatment. The graphs in Figures 4 A-D show the results of these analyses. None of the treatments had an effect on testicular expression of ER α (Figure 4A) at any time point.

Unlike ER α , ER β a did show time-dependence in its expression following treatment (Figure 4B). Both 2.5 mg/kg and 10 mg/kg MXC induced expression of ER β a significantly higher than E₂, but none of the treatments were different from vehicle. At 48 h post-treatment, only the 25 mg/kg MXC group was significantly higher than the vehicle (32.1-fold). By 72 h, E₂ caused a significant decrease (to 10% of vehicle) in the expression of ER β a, while none of the MXC treatment groups were significantly different from the level of the control.

ER β b (Figure 4C) was up-regulated equally by each of the three MXC treatments compared to vehicle by 24 h (about 7.9-fold). By 48 h, 25 mg/kg MXC was able to stimulate expression of this gene (11.0-fold) and this effect was not different from 2.5 mg/kg MXC. After 72 h, ER β b was again up-regulated by the MXC treatments compared to E₂, but this effect was not different from vehicle.

The expression of StAR (Figure 4D) was down-regulated by E₂ alone at 24 h (10.75-fold). After 48 h, all treatment groups were about equal in the expression of StAR. Then at 72 h, all treatments caused a down-regulation in the expression of StAR compared to vehicle (11.6% of vehicle across the treatment groups).

MXC and E₂ Affect ER Expression Differently in Liver and Testes

Figure 5 shows the results of side-by-side comparisons of the fold-changes from vehicle for each of the LMB ERs in the liver and testes at the 24 h time point. For ER α , E₂ stimulated expression in the liver, while it decreased expression in the testes. Both 2.5 mg/kg and 10 mg/kg MXC seemed to cause similar levels of increase between these two tissues. The highest dose of MXC gave a pattern of increasing ER α expression, in the liver while not really affecting expression in the testes. ER β a showed the same pattern of change in expression by the treatment as seen for ER α . ER β b expression was more highly stimulated by E₂ and 25 mg/kg MXC in the liver than in the testes, while both 2.5 mg/kg and 10 mg/kg MXC caused approximately equal changes.

There is a relatively linear dose response effect with MXC on the expression of the three estrogen receptors in the liver. However, in the testis, the dose response curve appears as an inverted U, where the lower concentrations increase ER expression above the level of the highest concentration of MXC. E₂ at 1 mg/kg also appears to have a negative effect on the transcription of the ERs α and β a, and only a small up regulation of ER β b in the testis.

Residues of MXC and Metabolites in Liver

Extraction and HPLC analysis of liver showed that MXC was present in the livers of bass dosed with 25 mg/kg at the three time points studied (Table 2). The concentrations found corresponded to between 0.1 and 0.2% of the administered dose. The mono-demethylated metabolite, OH-MXC was found in all liver samples, whereas the bis-demethylated metabolite, HPTE was detected in only one liver sample, taken at 24 h. The efficiency of extraction of MXC and metabolites from spiked liver samples was $91 \pm 1\%$ (mean $\pm$ S.D. $n = 3$). The values shown in Table 2 are not corrected for recovery. The HPLC method separated HPTE, OH-MXC and MXC from other components in the liver, with retention times of 13.3 min, 17.1 min and 20.9 min, respectively under the conditions used.

Discussion

The goal of this study was to measure changes in the expression of some genes related to reproduction following exposure to the pesticide MXC and to compare its effects to E₂ in male LMB liver and testes. Initial trials to test the effects of three different concentrations of MXC on gene expression involved challenging the LMB with concentrations of MXC at a higher range of doses (250 mg/kg, 100 mg/kg, and 25 mg/kg) which were based on the

concentration of 254 mg/kg previously published to be effective for channel catfish (Schlenk et al., 1997). However, a few hours following treatment, all of the fish treated with 250 mg/kg (22/22) were dead, while with 100 mg/kg, all but three of the fish died (19/22), and with the 25 mg/kg treatment, only three fish died (3/21). Individuals treated with vehicle (DMSO) or 1 mg/kg E₂ survived. From these data we conclude that LMB are more sensitive to MXC than channel catfish. Before they died, the fish appeared to have trouble with orientation (swimming erratically or upside down). In addition, they appeared to be having some breathing difficulties. It is possible that DMSO allowed rapid release of the MXC into the tissues resulting in the acute toxicity, as these symptoms are known signs of acute MXC exposure. Therefore we tested the response of the fish using other vehicles and determined that the combination of acetone:sesame oil (6.25%:93.75% v/v) was adequate for this experiment, as no acute toxicity was seen with this mixture.

It has previously been shown that the male LMB liver responds to E₂ with respect to the expression of ERs α and β and Vtg 1 (Sabo-Attwood et al., 2004). This study is the first to show that MXC also alters expression of ERs and Vtg I in the liver in the LMB. ER α was up-regulated by E₂ and by 25 mg/kg MXC after 24 and 48 h and by 10 mg/kg MXC after 48 h. The expression of the two ER β s were similar to one another, where they were each stimulated by all treatments compared to vehicle by 24 h and returned to the level of control by 48 h. The major biomarker in male fish for the estrogen response is the expression of Vtg 1 in the liver. We found that MXC at all concentrations was able to up-regulate the expression of Vtg 1 by 24 h. This response is likely largely dependent on existing ERs in the liver, however, as we have demonstrated in the past (Bowman et al. 2002), even further increase in Vtg expression can be associated with an increase in expression of ER α , as seen for the E₂ and 25 mg/kg MXC treatments. We speculate that these data suggest that the sensitivity of the ER α promoter to MXC is less than that of the Vtg 1 promoter, since all concentrations of MXC were able to increase Vtg at the 24 h time point, while only E₂ and the highest concentration of MXC (25 mg/kg) increased ER α at this time point.

The much greater transcriptional activation of the ER β s in the liver by 25 mg/kg MXC compared to 1 mg/kg E₂, suggests that MXC is more potent than E₂ for these two genes. These effects lasted through 24 h for ER β _a and 48 h for ER β _b. Alternatively, it is possible that the differences are due to the biological half-lives of the two compounds. The half life for E₂ is relatively short in fish; for example in fathead minnow (*Pimephales promelas*) it is about 2-4 h (Korte et al., 2000). The half-life of MXC in LMB has not been reported, but is likely to be longer than that of E₂. In this study, the livers of the LMB treated with 25 mg MXC/kg contained residues of MXC for at least 72 h following treatment. The residues included unchanged MXC and smaller concentrations of OH-MXC and very little, if any HPTE. It is likely that the OH-MXC and HPTE metabolites were formed in all MXC-treated fish, and then eliminated as the glucuronide conjugates. Studies with channel catfish showed that MXC was readily demethylated in hepatic microsomes forming OH-MXC and HPTE (Stuchal et al., 2006). Both OH-MXC and HPTE were substrates for glucuronidation in channel catfish liver (James et al., 2006).

A longer biological half life for MXC compared to E₂ may explain a decreased or delayed estrogenic effect, especially since the demethylated metabolites are more potent than the parent compound with the LMB ERs (Blum et al., 2007). This may explain the delayed stimulation of ER α seen with treatment with 10 mg/kg MXC compared to E₂. But, it may also be possible that MXC regulates the ER β s through a mechanism that does not require the ERs. It is not clear in fish how the promoters for the ER β s are regulated, whether they can be self-regulated or cross-regulated, or if they are regulated through ER activity at all. This appears to be true for the human ERs as well upon inspection of the current literature. There are many reports showing cooperativity between the receptors themselves regulating other

genes as both homo- and hetero-dimers (Hall and McDonnell 1999; Sabo-Attwood et al., 2007; Tremblay et al., 1999), but nothing on how they regulate their own promoters directly, and therefore their regulation may be through other mechanisms of action.

We found that the expression of mRNA for CYP3A isoform 68 was increased by all treatments in the liver by 24 h after treatment. There was similarly an increase in CYP3A activity with all treatments by 24 h, but this increase occurred in a pattern that was different from the gene expression levels. The CYP3A activity measured was likely the sum of activities from multiple isoforms, including for example isoform 69 which has been shown to be present in the liver of LMB (Barber et al., 2007). Further studies assessing the specific roles of the individual CYP3A isoforms need to be performed. It was interesting to see that CYP3A68 is quickly up-regulated in the liver within 24 h of treatment. This appears to match the regulation of the ER β s (ER β b in particular). The CYP3A isoforms are important for metabolizing endogenous steroids and other lipid hormones (Guengerich, 1991) as well as xenobiotic agents (Schuetz et al., 1998; James et al., 2005; Meucci and Arukwe, 2006; Mortensen and Arukwe, 2006) and their promoter organization and regulation in mammals have been partially characterized (Anakk et al., 2003), but there is no information on the promoters in LMB. CYP3A has been shown to be upregulated by MXC in rats (Mikamo et al., 2003) and by other endocrine disruptors in MCF-7 and HepG2 human cell lines (Coumoul et al., 2002) through the pregnane-x-receptor (PXR). However, it is not clear whether it is the parent compound or the bioactivated metabolites that are causing the up-regulation of CYP3A.

MXC is readily metabolized into OH-MXC and HPTE in the liver. The metabolites have been shown to be the main ligands for estrogenic responses in mammalian systems exposed to MXC. Gaido et al. (1999) showed that for both human and rat ER α , HPTE is a potent agonist and in combination with E₂, it is further able to increase receptor activity. At the same time, HPTE is a potent human ER β antagonist, capable of decreasing ER β activity. Both OH-MXC and HPTE have been detected in trout (Ohyama et al., 2004), channel catfish (Stuchal et al., 2006), and in small amounts in this study, suggesting that the estrogenic effects seen, for example the up-regulation of Vtg 1 and the ERs, could be occurring as a consequence of the metabolites, rather than the parent compound. The LMB ERs, however, respond differently to MXC and its metabolites compared to the mammalian ERs. *In vitro* transcriptional activation studies with LMB ERs showed that the parent compound MXC, as well as its metabolites are capable of activating all three of the receptors (Blum et al., 2007). In that study, we transfected the LMB ERs into HepG2 cells (human hepatocellular carcinoma) and tested their transactivation with MXC and the MXC metabolites and found that they behave quite differently from the human and rat ERs (Gaido et al., 1999). For the LMB ERs, the potency of the ligand increased with the degree of demethylation for both ER α and ER β b. However, the opposite was found for ER β a. The efficacy of these ligands was also always highest for ER β b, followed by ER α , while ER β a activity, although significantly stimulated, was never greater than 2-fold with any of the ligands (Blum et al., 2007).

In the testes, stimulation of expression of the measured genes is different from that seen in the liver. The expression of ER α is unaffected by E₂ or by MXC at any time point measured. In contrast, the ER β s are responsive to MXC, with no significant difference among the doses at 24 h. After 48 h, ER β a and ER β b are significantly up-regulated, but only by 25 mg/kg MXC. It is possible that the two ER β s are regulated in similar fashion in the testes based on their patterns of expression influenced by MXC. These results are consistent with the idea that the genes arose as the result of gene duplication sometime during the evolution of the line of teleost fishes (Hawkins et al., 2000).

The expression of StAR was quite different from the other genes. MXC did show an effect on its expression, but regardless of dose, it took 72 h to see the effect. The reason for the time delay is unclear, but may be due to a secondary response resulting from a metabolite of MXC, or through alteration of steroid biosynthesis pathways through certain feedback loops. Alternatively, this effect can be due to altered activity of other transcription factors. In comparison to MXC, E₂ was able to decrease the expression of StAR after only 24 h. After this time point, the response leveled off where there were no differences by treatment at 48 h, but StAR was down-regulated again at the 72 h time point. In other work from our lab, it was discovered that there is an estrogen receptor element half-site in the distal part of the promoter of LMB StAR (Kocerha, 2005). This half-site ERE may be involved in the regulation of this pathway, but to date no direct interaction between the LMB ERs and the StAR promoter has been shown.

In conclusion, we have found that MXC stimulates estrogen-like responses in the liver, similar to the action of E₂. In addition, MXC increased CYP3A68 mRNA expression and activity. The effects from a single injection are relatively short-lived; suggesting that metabolism of MXC may efficiently reduce the levels of toxicant in fish. The effects in the testis were quite different, with significant up regulation of mRNAs for the ER β s and a down regulation of StAR. Taken together these data suggest that MXC works as an endocrine disruptor and could affect reproduction.

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Figure 1.

Structures of DDT and MXC. DDT and MXC are similar molecules which vary in two positions. The underlined chlorine atoms in DDT were replaced with the underlined methoxy groups in MXC. This modification decreases the environmental and biological half-life of MXC when compared to DDT.

Figure 2.

The effects of E₂ or MXC on gene expression in the liver. Data presented are the means $\pm$ SEM of the log₁₀ (copy number) for each gene normalized to the log₁₀ (copy number)18S rRNA present in the sample. Differences between treatments within each time point were determined using Duncan's multiple range test. The line graphs next to each of the bar graphs show the fold-change from vehicle over time by subtracting the log₁₀ of the vehicle from each treatment. The zero time point used the means of untreated fish. Bars with different letters are significantly different. A) ER α expression. B) ER β α expression. C) ER β β expression. D) Vtg I expression. E) CYP3A68 expression.

Bars ■ Vehicle ▨ 1 mg/kg E₂ ▩ 2.5 mg/kg MXC ▪ 10 mg/kg MXC ■ 25mg/kg MXC
 Lines ● Vehicle ○ 1 mg/kg E₂ ▼ 2.5 mg/kg MXC ▲ 10 mg/kg MXC ■ 25 mg/kg MXC

Figure 3.

The effects of E₂ or MXC on the activation of CYP3A activity after 24 h. Data presented are the mean $\pm$ SEM of the total CYP3A activity after 24 h of treatment from liver microsomes of LMB as described in the Materials and Methods. Differences between the treatment groups were determined using Duncan's multiple range test with $\alpha=0.05$. Bars with different letter are statistically different.

Figure 4.

The effects of E₂ or MXC on gene expression in the testes. Data presented are the means $\pm$ SEM of the log₁₀ (copy number) for each gene normalized to the log₁₀ (copy number) 18S rRNA present in the sample. Differences between treatments within each time point were determined using Duncan's multiple range test. The line graphs next to each of the bar graphs show the fold-change from vehicle over time by subtracting the log₁₀ of the vehicle from each treatment. The zero time point used the means of untreated fish. Bars with different letters are significantly different. A) ER α expression. B) ER β expression. C) ER β expression. D) StAR expression.

Bars ■ Vehicle ▨ 1 mg/kg E₂ ▩ 2.5 mg/kg MXC ▪ 10 mg/kg MXC ■ 25 mg/kg MXC
 Lines —●— Vehicle —○— 1 mg/kg E₂ —▼— 2.5 mg/kg MXC —▲— 10 mg/kg MXC —■— 25 mg/kg MXC

Figure 5.

Comparison of the change in gene expression for each of the LMB ERs between liver and testes. The effects of E₂ or MXC are tissue and dose-dependent. Values are the mean fold-change from vehicle on the log₁₀ scale. Data are means $\pm$ SE of the 18S rRNA normalized values.

Table 1

Primers used for quantitative real time PCR

Gene	Forward Primer	Reverse Primer	Conc (nM)	Ref
18S	CGGCTACCACATCCAAGGAA	CCTGTATTGTTATTTTCGTCACCT	400	
ER α	CGACGTGCTGGAACCAATGACAGAG	TCCGGTCACTGATGATTTCTCCTCCA	400	Sabo-Attwood et al., 2004
ER β a	GTGACCCGTCTGTCCACA	TCTGGGGCTAGTGCAGGAGA	200	Sabo-Attwood et al., 2004
ER β b	CCGACACCGCCGTGGTGACTC	AGCGGGGCAAGGGAGCCTCAA	200	Sabo-Attwood et al., 2004
Vtg 1	AAGCCCATCCAGGATCTC	GCTGCAGTGCCATGTATG	900	Sabo-Attwood et al., 2004
StAR	ACCCCTCTGCTCAGGCATTT	GGGCTCCACCTGCTTCTTTG	400	Kocerha 2005, Garcia-Reyero et al., 2006
CYP3A68	TGCACCGGGACCCTGAT	TGCTGAACCTCTCAGGTTTGAA	400	Garcia-Reyero et al., 2006, Barber et al., 2007

Table 2

Methoxychlor metabolites detected in largemouth bass liver following IP injection with 25 mg/kg MXC. Values shown are pmol/g wet wt, mean $\pm$ S.E., n=3, except as indicated.

Metabolite	24 h	48 h	72 h
HPTE	N.D. – 96 ^a	N.D	N.D.
OH-MXC	87 $\pm$ 36	45 $\pm$ 26	154 $\pm$ 111
MXC	2720 $\pm$ 960	3370 $\pm$ 2250	4350 $\pm$ 1060

^aN.D. Not detected (<20 pmol/g). HPTE was not detected in two of the three 24 hr samples. The recovery of MXC, OH-MXC and HPTE from spiked liver samples was 91 $\pm$ 1%. The results shown are not corrected for recovery.