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Exposure to *p,p'*-DDE or dieldrin during the reproductive season alters hepatic CYP expression in largemouth bass (*Micropterus salmoides*)

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Abstract

Largemouth bass (LMB) in Central Florida living on sites with high levels of organochlorine pesticides (OCPs) have exhibited poor reproductive success and altered steroid profiles. The mechanism underlying these changes is unknown, however changes in the rate of steroid metabolism could alter steroid homeostasis. Members of the CYP2 and CYP3A families play a significant role in the metabolism of many xenobiotics and endogenous compounds, including sex steroids. Therefore, the goal of this study was to identify members of the CYP2 and CYP3A families in LMB and characterize the effects of OCP exposure on their expression. Full-length clones of two CYP3A isoforms were obtained from LMB liver, CYP3A68 and 3A69, which exhibited significant sequence divergence. Full-length clones for CYP2N14 and CYP2P11 were also obtained from LMB liver. Steady-state mRNA levels of each of these CYPs increased in both sexes between early reproductive phase (December) and peak reproductive phase (March). Expression of CYP3A68 and CYP2P11 was sexually dimorphic during peak reproductive phase with 2-fold higher expression in females and males, respectively. Foodborne exposure to 46 ppm *p,p'*-DDE or 0.8 ppm dieldrin for 30 days did not have a significant effect on expression of CYPs. However, 4 months exposure to *p,p'*-DDE induced CYP3A68 and 3A69 expression in both sexes, while dieldrin produced weak induction of CYP3A68 and suppressed CYP3A69 expression in females, but had no effect on males. Neither *p,p'*-DDE nor dieldrin significantly altered the expression of CYP2P11 or CYP2N14. This work demonstrates that there are significant changes in CYP expression that occur during LMB reproduction which can be modified by exposure to OCPs.

Keywords

Cytochrome P450; Organochlorines; Largemouth bass

1. Introduction

The cytochrome P450 (CYP) superfamily of heme-containing enzymes catalyzes Phase I biotransformation of endogenous and xenobiotic compounds, including fatty acids, steroids, drugs, and environmental contaminants. Over 1900 CYP isoforms with overlapping substrates have been identified in animals (P450 Nomenclature Committee, 2006). Most of these have been described in mammals and only recently have substantial numbers of CYPs been identified in fish. Because activity of these enzymes is often the determining factor in susceptibility of organisms to toxicity, knowledge of substrate specificity and regulation of

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CYPs has been useful in predicting toxicity across species. Unfortunately, mammalian models of CYP metabolism have not always successfully transferred to fish (Miranda et al., 1998). To obtain a better understanding of P450 regulation and activity in fish, the database of available sequences in fish needs to be expanded.

The largemouth bass (*Micropterus salmoides*, LMB) is a member of the sunfish family (Centrarchidae) that is widely distributed in fisheries throughout the Continental United States. Due to their position near the top of the food chain, LMB accumulate many lipophilic contaminants, such as organochlorine pesticides (OCPs), which may adversely impact the fish. In central Florida, LMB introduced to reclaimed farmland around Lake Griffin in Florida have exhibited poor reproduction and population declines (Douglas and Benton, 1996) that correlates with altered levels of circulating sex steroids (Gross et al., 1995). DDT residuals (primarily *p,p'*-DDE), toxaphene, chlordanes and dieldrin are the major OCP contaminants found in LMB living in the affected sites (Marburger et al., 2002). Exposure to OCPs is known to alter CYP expression (You et al., 1999) which in turn can change the rate of steroid metabolism and potentially alter steroid homeostasis (McMaster et al., 1991; Wilson and LeBlanc, 1998).

In fish, members of the CYP2 and CYP3A families play a major role in the metabolism of xenobiotics and endogenous compounds, including steroids (Miranda et al., 1989; Brian et al., 1990; Thibaut et al., 2002). Therefore, the goal of the present study was to identify LMB CYP2 and CYP3A isoforms, characterize their normal expression during the reproductive cycle and determine the effect of OCP exposure on their expression. To this end, we obtained full-length clones of CYP3A68, CYP3A69, CYP2N14, and CYP2P11 from LMB liver, examined normal hepatic expression in male and female fish during the early and late reproductive phase, and determined the effect of subchronic *p,p'*-DDE and dieldrin exposure on their expression.

2. Materials and methods

2.1. Molecular cloning of CYPs

Total RNA was isolated from largemouth bass (LMB) liver using Trizol (Invitrogen, Carlsbad, CA). Degenerate primers were designed using known cytochrome P450 3A (CYP3A) and 2 (CYP2) protein sequences from mammals, fish, and one reptile. The protein sequences were aligned and highly conserved regions were used to design the degenerate primers (Table 1). Reverse transcription (RT) was performed with random hexamer primers (Invitrogen, Carlsbad, CA) using the RETROscript™ Kit from Ambion (Austin, TX) according to the manufacturer's protocol.

PCR was performed on the resulting cDNA using the indicated degenerate primers (Table 1). PCR products were gel purified and ligated into pGEM-T Easy Vector (Promega, Madison, WI) for *E. coli* JM109 competent cell transformation. Recombinant plasmids were isolated and sequenced at the University of Florida DNA Sequencing Core Laboratory. Two distinct CYP3A sequences and two distinct CYP2 sequences were present in the recombinant plasmids. Plasmids containing each insert were sequenced at least three times to obtain accurate consensus sequence.

2.2. RLM-RACE PCR

In order to obtain full-length cDNA of LMB CYPs, RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) of both 5'- and 3'-end was conducted. RACE was performed using the FirstChoice™ RLM-RACE Kit purchased from Ambion (Austin, TX) according to the manufacturer's instructions. PCR conditions for amplification of the LMB CYP3A68, CYP3A69, and CYP2N14 cDNA ends were 35 cycles of denaturation at 94 °C for 30 s, primer

annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The amplification of LMB CYP2P11 was carried out as above, but with an increase in primer annealing temperature to 65 °C. The RT and PCR reaction mixtures were prepared as described above. Nested PCR was used to obtain good yield of 5'-RACE products. In these reactions, the initial PCR amplification was performed with the outer primers (Table 1). One microliter of the first PCR reaction was used as template for nested PCR amplification with inner primers (Table 1).

2.3. Quantitative PCR analysis

Expression of individual CYPs was quantified by real-time PCR (qPCR) using the gene specific qPCR primers listed in Table 1 and SYBR Green detection (Bio-Rad, Hercules, CA). Expression of each gene was quantified in the livers of three to five individuals for each treatment group. Duplicate reactions were performed for each sample and the mean C_t value was used to determine relative expression. Relative changes in steady-state message level for the samples treated with *p,p'*-DDE and dieldrin were calculated using the $\Delta\Delta C_t$ method using 18S rRNA as the normalizing gene. 18S rRNA was quantified using the Human 18S TaqMan Assay from Applied Biosystems, Inc. (Foster City, CA).

In order to calculate the number of copies for each gene, standard curves were generated using different amounts of plasmid containing the specific cDNA inserts as template. Plasmid concentration was determined by absorbance at 260 nm. To convert mass of plasmid to copy number, the following calculation was used:

$$M = (\text{DNA concentration in g / ml} / \text{plasmid size in bp}^a \times 660^b) \\ \times \times 6.022 \times 10^{23} \text{ copies / mol}$$

where M is the copy number/μl of template, a is the total plasmid size including vector and insert and b is the average molecular weight of a single base pair.

The calculated number of copies for each gene was normalized to amount of RNA in each reaction for control samples.

2.4. Effect of *p,p'*-DDE and dieldrin treatment on CYP expression

LMB with a mean body weight of 159 g (20–30 cm) were purchased from American Sportfish Hatchery (Montgomery, AL). Fish were housed at the USGS Caribbean Research Center (Gainesville, FL) in 6000 l outdoor concrete raceways under ambient conditions. 100 fish were placed in each tank. Tanks were provided with constant flow of pond water and forced air aeration. All procedures involving live fish were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

LMB were exposed to either *p,p'*-DDE or dieldrin by dissolving the compounds in menhaden oil which was used to top-dress Silvercup floating pellets (Ziegler Bros., Murray, UT). Representative samples of the treated feed were analyzed by GC–MS in the University of Florida Analytical Toxicology Core Laboratory as previously described (Gelsleichter et al., 2005). *p,p'*-DDE treated feed contained 46 μg *p,p'*-DDE/g feed and dieldrin treated feed contained 0.8 μg dieldrin/g feed. Control feed was also dressed with menhaden oil. All fish were fed at 1% of their average body weight and feed amounts were adjusted every 30 days to compensate for growth. Experiments began in November and terminated in March to coincide with the onset and peak periods of reproductive activity in LMB. Total duration of feedborne exposure was 120 days for *p,p'*-DDE and 136 days for dieldrin. Water temperatures varied from 12.2 °C to 22.4 °C during the 4-month exposure period. At each timepoint, 12 fish were euthanized and liver samples were snap frozen in liquid nitrogen and stored at –80 °C until analysis. RNA was isolated and real-time PCR was performed as described above to quantify

changes in steady-state levels of mRNA for each CYP. Blood collected from the caudal vein in heparinized syringes was centrifuged to obtain plasma, which was frozen for steroid analysis. Whole carcasses with liver and gonads removed were ground and mixed. Representative samples were analyzed for *p,p'*-DDE and dieldrin by GC-MS in the University of Florida Analytical Toxicology Laboratory as previously described (Gelsleichter et al., 2005).

2.5. Steroid analysis

Estradiol and 11-ketotestosterone (11-KT) were determined in LMB plasma by radioimmunoassay using the method described by Muller et al. (2004). Estradiol antibody was purchased from ICN Biomedical (Costa Mesa, CA) and 11-KT antibody was purchased from Helix Biotech (Richmond, BC, Canada).

2.6. Phylogenetic analyses

Phylogenetic comparisons were performed using LMB 3A68, LMB 3A69, Trout 3A27 (O42563), Trout 3A45 (AAK-58569), Medaka 3A38 (AAG35209), Medaka 3A40 (Q98T91), Chicken 3A37 (CAB62060), Ball Python 3A42 (AAG33639), Killifish 3A30 (AAF14117), Killifish 3A56 (AAN38837), Pufferfish 3A47, Pufferfish 3A48, Pufferfish 3A49, Pufferfish 3B1, Pufferfish 3B2 (all from P450 Nomenclature Committee, 2006), Zebrafish 3A65 (AY452279) and human 3A4 (P08684). Amino acid sequences for these isoforms were aligned using Clustal W in the Phylip format. The phylogenetic tree was constructed using Phylip (v. 3.65). Within Phylip, the Seqboot tool was used to generate 500 bootstrapped data sets (Felsenstein, 1985) which were analyzed by the Protpars tool to determine the most parsimonious tree. The Consense tool was used to identify the majority rule consensus tree and compile the frequency with which two isoforms were separated. CYP3B1 and 3B2 were defined as outgroups to root the tree. Outputs from Phylip were edited for publication using TreeView (Page, 1996).

2.7. Statistical analysis of data

Changes in copy number of each CYP isoform in control animals at the two timepoints were analyzed by two-way analysis of variance (ANOVA) with sex and month as the variables. Effects of contaminant treatment on CYP expression was analyzed by three-way analysis of variance (ANOVA) with sex, exposure duration and contaminant as the variables (Sigma STAT 2.0, Jan-del Scientific Software, San Rafael, CA). Main effects and all two- and three-way interactions were analyzed. Post hoc comparisons for all significant terms were performed with Tukey's HSD and *p* values <0.05 were considered to be significantly different.

3. Results

3.1. Cloning of CYPs

PCR amplification of male LMB liver cDNA with degenerate primers designed against conserved regions of CYP2 isoforms resulted in identification of two distinct cDNA clones of 666 bp. Sequencing of these products revealed two distinct CYP sequences, one of which was homologous with CYP2P and one with CYP2N. Following 5' and 3'-RACE, full-length sequences were obtained for each of these products. The CYP2P clone was 1992 bp in length and contained an open reading frame between nucleotides 121 and 1568 that encodes a protein of 498 residues with a predicted molecular mass of 56,901 Da. This protein contains a predicted heme-binding peptide with the sequence FSAGKRVCLGEQLA including the invariant cysteine at residue 445. This P450 has been designated CYP2P11 by the P450 Nomenclature Committee (GenBank Accession # DQ786405). The CYP2N clone was 1968 bp in length and contained an open reading frame between nucleotides 158 and 1651 that encodes a protein of 497 residues with a predicted mass of 56,392 Da. This protein contains a predicted heme-

binding peptide with the sequence FSAGKRVCLGQGLA with the invariant cysteine at residue 444. This P450 has been designated CYP2N14 by the P450 Nomenclature Committee (GenBank Accession # DQ786404).

PCR amplification with degenerate primers designed against conserved regions of CYP3A isoforms resulted in identification of two distinct cDNA clones of 886 bp. 5' and 3'-RACE was used to obtain full-length sequences for each of these products. One CYP3A clone was 1809 bp in length and contains an open reading frame between nucleotides 46 and 1561 that encodes a protein of 504 residues with a predicted mass of 58,042 Da (Fig. 1). This protein contains a predicted heme-binding peptide with the sequence FGAGPRNCIGMRFA with the invariant cysteine at residue 445. This P450 has been designated CYP3A68 by the P450 Nomenclature Committee (GenBank Accession # DQ786406). The other CYP3A clone was 2462 bp in length and encodes a protein of 497 residues with a predicted mass of 57,140 Da (Fig. 1). This protein contains a predicted heme-binding peptide with the sequence FGIGPRNCLGMRFA with the invariant cysteine at residue 443. This P450 has been designated CYP3A69 by the P450 Nomenclature Committee (GenBank Accession # DQ786407).

LMB CYP3A68 shares 71–80% nucleotide sequence identity with CYP3A isoforms identified in other fish and 64% identity with human CYP3A4. LMB CYP3A69 shares only 62–66% identity with CYP3A isoforms from other fish (Table 2A). CYP3A68 and 3A69 share only 66% sequence identity with each other (Fig. 1, Table 2A). Of interest, the putative substrate recognition sites in CYP3A68 and 3A69 differ significantly. Of the 76 amino acids present in these recognition sites, 28 differ between 3A68 and 3A69 (Fig. 1). LMB CYP2P11 shares ~79% identity with CYP2P isoforms identified in killifish (*Fundulus heteroclitus*) (Table 2B). LMB CYP2N14 shares 77–79% identity with CYP2N isoforms from the killifish (*F. heteroclitus*) and pufferfish (Table 2C).

Phylogenetic comparisons of LMB CYP3A68 and 3A69 were performed using other members of CYP3A identified in fish as well as chicken, ball python and human. Results of these analyses show that both LMB 3A68 and 3A69 cluster with other fish from the superorder acanthopterygii. However, LMB CYP3A68 is more closely related to CYP3A isoforms found in killifish (cyprinodontiformes) and medaka (beloniformes), while LMB CYP3A69 is most similar to CYP3A47 from pufferfish (tetraodontiformes) (Fig. 2).

3.2. CYP mRNA levels in untreated male and female LMB during early and peak reproductive phases

To determine if these CYPs are differentially expressed in male and female LMB during various stages of reproduction, steady-state mRNA levels of these CYP isoforms were examined as a function of sex and stage of the reproductive cycle. Hepatic mRNA levels were quantified by real-time PCR in untreated fish at experimental days 30 (December) and 120 (March) which correspond to early reproductive and peak reproductive times, respectively. In December, there were no statistically significant differences in hepatic expression of any of the CYP isoforms examined (Fig. 3). In March, the steady-state mRNA levels of each of the isoforms increased in both sexes compared to December, though the magnitude of increase varied with sex and isoform. This led to sexually dimorphic expression of some isoforms at this stage of the reproductive cycle. CYP3A68 expression was approximately 2-fold higher in females than males, but CYP3A69 transcripts were approximately 50% more abundant in male. CYP2P11 was nearly twice as abundant in male LMB livers. CYP2N14 was highly expressed in both male and female LMB livers (Fig. 3).

3.3. Effect of *p,p'*-DDE and dieldrin exposure on CYP expression in LMB liver

Dietary exposure to *p,p'*-DDE and dieldrin produced significant increases in body content of these chemicals. Following dietary exposure to 46 ppm *p,p'*-DDE for 120 days or 0.8 ppm dieldrin for 136 days, LMB carcasses contained 7888.5 ± 1666 ng *p,p'*-DDE/g wet weight ($93,437 \pm 23,720$ ng *p,p'*-DDE/g lipid) and 265.3 ± 49.6 ng dieldrin/g wet weight (2955 ± 746 ng dieldrin/g lipid), respectively. Control fish contained 94 ± 163 ng *p,p'*-DDE/g wet weight and 1.8 ± 1.1 ng dieldrin/g wet weight. No significant differences were observed in contaminant burden between male and female fish.

Following 4 months of exposure, circulating levels of estradiol in female LMB plasma were 637 ± 139 , 308 ± 116 , and 152 ± 43 pg/ml in control, *p,p'*-DDE, and dieldrin treated fish, respectively. 11-KT levels in male LMB plasma were 956 ± 62 , 1033 ± 307 , and 252 ± 61 pg/ml in control, *p,p'*-DDE, and dieldrin treated fish, respectively.

Significant effects of sex, exposure duration, and contaminant as well as their interactions were observed on CYP3A expression. Exposure to *p,p'*-DDE or dieldrin for 30 days did not produce significant changes in steady-state message levels of any of the CYP isoforms analyzed when compared to control. The effects of *p,p'*-DDE and dieldrin on expression of CYP3A68 and 3A69 tended to differ in males and females, though differences were not significant (Fig. 4A). Exposure of LMB to *p,p'*-DDE for 4 months during the reproductive phase increased hepatic expression of CYP3A68 and 3A69 in males and females by 1.7–2.9-fold. Exposure to dieldrin during this period increased hepatic expression of CYP3A68 by 2.1-fold but decreased CYP3A69 expression by 2.5-fold in females. Hepatic expression of CYP3A68 and 3A69 in males were not affected by exposure to dieldrin. Four months exposure to *p,p'*-DDE or dieldrin did not produce significant changes in the hepatic expression of CYP2P11 or CYP2N14 in LMB liver (Fig. 4B).

4. Discussion

4.1. Identification of CYPs in LMB liver

Our results demonstrate that largemouth bass express two isoforms of CYP3A in the liver. In most teleost fish with multiple CYP3A isoforms, including trout, killifish and medaka, there is greater than 90% sequence identity between CYP3A isoforms (Kullman and Hinton, 2001; Hegelund and Celander, 2003), however, LMB CYP3A68 and 3A69 share only 66% nucleotide sequence identity. A similar situation exists in pufferfish (*Takifugu rubripes*) where CYP3A47 is divergent from other CYP3A isoforms present in that fish (CYP3A48 and 3A49). The analysis strongly supports the presence of divergent CYP3A isoforms in LMB (CYP3A69) and pufferfish (CYP3A47). CYP3A47 was not included in previous CYP3A alignments due to its divergence (McArthur et al., 2003), however the presence of a similar isoform in another species indicates that there may be a group of CYP3A isoforms in some fish lineages that differ significantly from prototypical CYP3A isoforms.

While most P450s share structurally important sites, such as heme-binding and oxygen-binding regions, substrate specificity of P450s is related to the amino acid sequence of variable regions known as substrate recognition sites (SRS, Gotoh, 1992). SRS were originally described for CYP2 but the model has been adapted for CYP3 (Harlow and Halpert, 1997). Using this model, there are considerable differences between the SRS of CYP3A68 and 3A69, especially in SRS 2 and 6 (Fig. 1). Previous work has shown that slight changes in the amino acid sequence of the SRS can have significant impacts on substrate specificity and regio-selectivity of metabolites. For example, positions 119, 305, and 373 appear to play prominent roles in metabolism of testosterone and progesterone by CYP3A4 (Khan and Halpert, 2000). These observations appear to hold true in the teleost CYP3A SRS as medaka CYP3A38 and

CYP3A40 differ in 12 positions in SRS 1, 3, and 6, including positions 119 and 479. Only 3A38 appears to make 16-beta hydroxytestosterone, while both isoforms produce 6-beta and 2-alpha hydroxytestosterone (Kashiwada et al., 2005). Based on this information, it is very likely that LMB CYP3A68 and 3A69 will have different substrate and regio-specificity as they differ at residues corresponding to positions 210, 370, 376, and 479 among others.

We also identified two CYP2 isoforms: CYP2N14 and CYP2P11. The CYP2N and CYP2P subfamilies have been described in killifish (Oleksiak et al., 2000, 2003) as well as other fish (P450 Nomenclature Committee, 2006). However, they appear to be restricted to fish or lower vertebrates as there are no mammalian orthologues. The best characterized activity for these CYP families is fatty acid metabolism, but CYP2N1 and 2N2 from killifish efficiently catalyze benzphetamine *n*-demethylation (Oleksiak et al., 2000). Steady-state mRNA levels of CYP2N14 were considerably higher than those of the other CYP isoforms examined. This is somewhat surprising given that CYP3A is typically the most abundant P450 isoform in fish liver (Hegelund and Celander, 2003). However, it is not known how mRNA levels are related to protein levels in LMB. Given the high level of expression, observed changes during the reproductive cycle and the recognized role of arachidonic acid metabolites on steroidogenesis (Wade and Van der Kraak, 1993), further investigation of the role of CYP2N in LMB reproduction is warranted.

4.2. Effect of reproductive phase on CYP expression in male and female LMB

Steady-state levels of hepatic CYP3A68, CYP3A69, CYP2N14, and CYP2P11 change during the reproductive cycle of LMB. Similar results have been observed for a variety of CYP isoforms in other fish, including female winter flounder (Gray et al., 1991), male sea bass (Vaccaro et al., 2005), striped mullet (Gorbi et al., 2005) and male turbot (Arukwe and Goksoyr, 1997). The mechanism responsible for modulation of CYP expression during the reproductive cycle is not clear, but is likely to be partially mediated by circulating steroid levels. During the period from December to March, LMB are preparing to spawn and there are dramatic increases in circulating estradiol, testosterone, and 11-ketotestosterone (Sepulveda et al., 2003; Sabo-Attwood et al., 2004). The lack of sex-related differences in CYP expression early in the reproductive cycle is consistent with the hypothesis that changes in circulating steroids help regulate expression of these CYP isoforms in LMB as circulating steroid levels are similar in both sexes are similar. In previous studies, treatment with exogenous estradiol decreased CYP3A protein expression in juvenile brook and rainbow trout, but testosterone exposure produced little effect (Pajor et al., 1990; Katchamart et al., 2002). It is likely that other factors involved in regulating reproduction, such as pituitary hormones and temperature, also play a role in regulating CYP expression during reproduction (Pajor et al., 1990).

Expression of many CYPs is sexually dimorphic, including some isoforms that are sex-specific. In fish, expression of CYP3A isoforms have been reported to be sex dependent in hepatic and extrahepatic tissues (Perkins and Schlenk, 1998; Hegelund and Celander, 2003). However, the patterns of sexual dimorphism appears to vary with CYP isoform and species, as male *F. heteroclitus* have higher CYP3A protein levels and activity than females, while immature female rainbow trout have higher CYP3A expression than males, and no sex differences have been observed in expression of CYP3A in human or scup (Gray et al., 1991; Lee et al., 1998). During the peak of the reproductive cycle, steady-state levels of CYP3A68 mRNA were 2–4 times higher in female LMB than in males, while levels of CYP3A69 and 2P11 mRNA were approximately 50% and 200% higher in males, respectively. The mechanisms underlying sexually dimorphic expression of CYPs is not fully understood though, growth hormone and steroids have been shown to regulate sex-specific expression of CYPs in mammals (Katchamart et al., 2002; Wiwi and Waxman, 2004).

4.3. Effect of OCP exposure on CYP expression

Changes in LMB hepatic CYP3A expression caused by exposure to *p,p'*-DDE or dieldrin are affected by duration of exposure. While short term exposure to *p,p'*-DDE or dieldrin did not affect hepatic CYP3A expression, subchronic exposure to *p,p'*-DDE and dieldrin altered expression of CYP3A68 and 3A69. Both chemicals bioaccumulate and the differences observed may reflect accumulation to some threshold concentration of the contaminants. However, the lack of effect of dieldrin in males following long-term exposure demonstrates that body burden is not the sole factor responsible as body burdens of dieldrin in males and females were equivalent despite the different responses.

p,p'-DDE and dieldrin produced different effects on CYP3A expression following subchronic exposure, though the mechanism responsible is not clear. *p,p'*-DDE and dieldrin have been shown to induce CYP3A expression by activation of the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) nuclear receptors in mammals. Though no functional homolog of CAR has been identified in fish, short-term treatment with *p,p'*-DDE induced PXR and CYP3A expression in the livers of juvenile Atlantic salmon (Mortensen and Arukwe, 2006). While PXR activation is a plausible explanation for the induction of CYP3A caused by *p,p'*-DDE, the varied responses caused by dieldrin exposure indicate that PXR activation is not the sole mechanism responsible.

As discussed above, steroid hormones regulate the expression of some CYPs so it is possible that *p,p'*-DDE and dieldrin modulated CYP expression by changing activation of steroid receptors. *p,p'*-DDE and dieldrin are both reported to have estrogenic and anti-androgenic activity, so it is possible that they acted directly at steroid receptors (Andersen et al., 2002). However, assays in Atlantic salmon, channel catfish and LMB indicate that dieldrin has little or no estrogenic activity (Tollefsen et al., 2003; Gale et al., 2004; Garcia-Reyero et al., 2006). Exposure to *p,p'*-DDE and dieldrin for 4 months altered endogenous levels of the major sex steroids in both male and female LMB, but there is no clear correlation between the changes of either androgens or estrogens and the changes observed in LMB hepatic CYP3A expression as a result of OCP exposure. The complexity of the transcriptional response of LMB CYP3A to dieldrin and *p,p'*-DDE exposure suggest that control of CYP3A expression is multi-factorial in LMB and may vary among sexes and isoforms.

Neither *p,p'*-DDE nor dieldrin exposure produced a significant change in the expression of CYP2N14 or CYP2P11 in LMB liver, indicating that these CYPs are controlled by mechanisms distinct from those involved in CYP3A. Previous work has demonstrated that CYP2P3 in killifish is modulated by tetraphorbol acetate (TPA) and fasting, potentially by actions on protein kinase C (Oleksiak et al., 2003).

5. Conclusions

Hepatic expression of CYP3A, CYP2N, and CYP2P isoforms in LMB varies with reproductive phase. During the peak of the reproductive cycle, there is sexually dimorphic expression of CYP3A68 and CYP2P11 at the message level. Exposure to *p,p'*-DDE and dieldrin altered steady-state mRNA levels of both CYP3A isoforms following subchronic exposure. This work demonstrates that long-term exposure to organochlorine pesticides during the reproductive phase of LMB produces compound and sex specific effects on CYP expression in LMB that coincide with changes in circulating steroid levels. The fact that responses differ between sexes and during the reproductive cycle highlights the need to examine effects of endocrine active chemicals in reproductively active animals.

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MGYFFFLSSAETWTLTLLVALITLIFVYVCWPFPGFTFKKMGVGPGRPIPFPGTGLAYKKGFMHF 60
MGYLPFLSSETWILLFTFICIFVTYGCWTFGTFEKLGIPGPKPLMYFGTIGRHNPPVYLA 60
: *:*: **:*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*

DSECFKKYKGTWGI F DGRQPVLCITDPAMIKTVLIKECYSLFTNRRNFRNLNGELYDAVSI 120
DNECAQKYGRVWGM YELRRPMLAVMDPDLKTLVKECFYFTNRRNIRLNGDLYDAVST 120
.:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***

AEDDQWRRIRSVLSPSFTSGRLKEMFDIMKHHSANLISSMKKTADKDEPLELKEFFGPGY 180
VEDDHWKRI RNILSPFFTSGHIKEMFSIMKHSHKLTASLQSKVHDDEVITIKDIFGPYS 180
.**:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***

MDVVTSTAFSVDIDSLNNPTDPFVANIKKMLKFDLFNPIFLFIAFFPFMAPILEKLEFSF 240
IDVMASCAFSVDMDTINNPSSPLITHASKLKFKS--KSLFLFQGCFFPLPLLELLGVSF 238
*:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***

FPKSVLDFFYAALKKIKANRETTKLKNRVDFLQLMINSQKNNDLSKVENDKGLNDHEILS 300
FPKTS TGFFKT VVEKIRAERNKSSQQNSGDVLQHMMNSQQANEPNKEKQNKGLTDHEITS 298
: .*::***:***:***:***:***:***:***:***:***:***:***:***:***:***

QAMIFLFAGYETTSSSLTFLAYNLARNPHVMKRLQEEVDATFPNKA PVEYQALMQMEYLD 360
QATMFVFAGYETSATTLVFLAYNLARNPEVMKHLQEEIESTFPNKGVPVQYEALMQMEYLD 358
*:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***

SVVNESLRLYPIAPRLERVA KATVEINGLVI PKGMVVMVPTWPLHRDPDLWPEPEEFKPE 420
SVVHESLRLYPPSPRLERI AKETVKISGITIPKDLVMTPVYALHRDPDLWPDPEEFKPE 418
::***:***:***:***:***:***:***:***:***:***:***:***:***:***

RFSKENKETIDPYTYMPFGAGPRNCIGMRFALVLMKLAVVEILQTYSFVCKETEPIEL 480
RFSKQNRQSINPYTYLPFGIGPRNCLGMRFALVMIKLALVEVLQNYSFVCEETEFPLKM 478
::***:***:***:***:***:***:***:***:***:***:***:***:***:***

DIQGLISPKRPIK LKLVPRSEPSN 504
DPLSLVGPLQPIK LKLVTR---- 497
.:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***

Fig. 1.

Amino acid sequence alignment of LMB CYP3A68 (upper) and CYP3A69 (lower). Substrate recognition sites (SRS) are indicated by shaded areas. Residues within the SRS that differ between sequences are denoted by bold text. The heme-binding domain is enclosed in a box. Below the alignment, asterisks (*) indicate sites that have identical residues, colons (:) indicate positions with conserved amino acid substitutions, Dots (.) indicate positions with semi-conserved amino acid substitutions.

Fig. 2. Consensus phylogenetic tree of CYP3A protein sequences. Aligned sequences were bootstrapped 500 times followed by maximum parsimony analysis. Numbers at branches indicate the frequency with which two groups joined by that branch were separated in the manner displayed.

Fig. 3.

Changes in hepatic steady-state mRNA levels of CYP isoforms in untreated LMB at various stages of reproduction. Asterisk (*) indicates that expression of an isoform differs significantly ($p < 0.05$) within a sex between December and March. Letter 'a' indicates that expression of an isoform differs significantly ($p < 0.05$) between sexes at a given time. Values are mean $\pm$ S.D. ($N = 3-5$).

Fig. 4. Effect of dietary *p,p'*-DDE or dieldrin on hepatic expression of CYPs in LMB following: (A) 1 month exposure to 46 ppm *p,p'*-DDE, (B) 1 month exposure to 0.8 ppm dieldrin, (C) 4 months exposure to 46 ppm *p,p'*-DDE, or (D) 4 months exposure to 0.8 ppm dieldrin. Values are $\log_2 \Delta\Delta C_t$ calculated for each gene and indicate the relative difference in expression between control and treated group. The corresponding fold change values are presented on the right axis. Asterisk (*) indicates that expression of an isoform differs significantly from control ($p < 0.05$) within a sex. Values are mean $\pm$ S.D. ($N = 3-5$).

Table 1

PCR primer sequences used in this study

Degenerate primers	
CYP3A	Forward primer: 5'-AARGARATGTTYGRIATHATGAA-3' Reverse primer: 5'-CCRAANGGCATRTAIRRTA-3'
CYP2	Forward primer: 5'-MGRRACTTTGGHMTGGG-3' Reverse primer: 5'-TCRTGGATVACWGCATC-3'
RLM-RACE primers	
CYP3A68	Outer 5'-RACE: 5'-CGGAGAGACTCGTTGACAACACTA-3' Inner 5'-RACE: 5'-TCTTCCTGCAGCCGTTTCAT-3' Outer 3'-RACE: 5'-CCAAAGGTATGGTCGTCATGGT-3'
CYP3A69	Outer 5'-RACE: 5'-AAGAGACACCCAGCAGTTCCAA-3' Inner 5'-RACE: 5'-CTTTGAAGCGTGGGTGATGA-3' Outer 3'-RACE: 5'-CCTGCAAGAGGAGATAGAATCCAC-3' Inner 3'-RACE: 5'-ACAGTGTGGTCCATGAGAGTCTGA-3'
CYP2N14	Outer 5'-RACE: 5'-ACTGAGCCCTCCAGCCACAAAAGT-3' Inner 5'-RACE: 5'-TGCTTTTGCCCATGCCAAAGTCCC-3' Outer 3'-RACE: 5'-AGCACTTGCCAGGTCCTCACAACA-3' Inner 3'-RACE: 5'-ACCTCCACCACTTTGTTGTGGGCT-3'
CYP2P11	Outer 5'-RACE: 5'-CGGGCACCCACTTCATCAGCCA-3' Inner 5'-RACE: 5'-GCGATCACCAACACCAGGCAGCA-3' Outer 3'-RACE: 5'-GCAAGGCAAGCCTTTTGATGCCCA-3' Inner 3'-RACE: 5'-AAAGCCTTGACCCGTCCTCACCGA-3'
qPCR primers	
CYP3A68	Forward primer: 5'-TGACCCGGGACCCTGAT-3' Reverse primer: 5'-TGCTGAACCTCTCAGGTTTGAA-3'
CYP3A69	Forward primer: 5'-GGCGAGCTGTGCATTCACT-3' Reverse primer: 5'-CGTGGGTGATGAGAGACTTG-3'
CYP2N14	Forward primer: 5'-AACGCCGTGGGCAACAT-3' Reverse primer: 5'-CGCTGTATTCAAACCGTTTCC-3'
CYP2P11	Forward primer: 5'-CCAGTGCTGTGTCCAACATCA-3' Reverse primer: 5'-CCCTGGTACTGCTGTGTCAGTGTAT-3'

Table 2

Nucleotide sequence similarity of LMB CYPs with CYPs from other species: (A) LMB CYP3A, (B) LMB CYP2P11 and (C) LMB CYP2N14

Species/accession	CYP	% Identity with LMB CYP3A68	% Identity with LMB CYP3A69
(A) LMB CYP3A			
Japanese Medaka/AF251272	3A40	80	65
Japanese Medaka/AF105018	3A	79	65
Killifish/AF105068	3A30	80	66
Killifish/AY143428	3A56	79	66
Rainbow Trout/U96077	3A27	75	65
Rainbow Trout/AF267126	3A45	76	65
Zebrafish/AY452279	3A56	71	62
Large mouth bass	3A68	—	66
Species/accession	CYP		% Identity with LMB CYP2P11
(B) LMB CYP2P11			
Killifish/AF117341		2P1	79
Killifish/AF117342		2P2	79
Killifish/AF117343		2P3	78
Species/accession		CYP	% Identity with LMB CYP2N14
(C) LMB CYP2N14			
Killifish/AF090434		2N1	77
Killifish/AF090435		2N2	77
Pufferfish/CR647983 ^a		cDNA	79

^aFull-length cDNA.