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Dietary exposure of largemouth bass to OCPs changes expression of genes important for reproduction

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Abstract

Dieldrin and *p,p'*-DDE are ubiquitous contaminants known to act as endocrine disruptors, causing impaired development and reproduction in fish and wildlife. In order to elucidate the mechanisms by which dieldrin and *p,p'*-DDE cause endocrine disruption in largemouth bass (*Micropterus salmoides*), fish were exposed subchronically through the diet to both contaminants. Following 120 days of exposure, *p,p'*-DDE decreased estradiol in females, but increased 11-ketotestosterone in both sexes. Dieldrin on the other hand, decreased estradiol and 11-ketotestosterone in both sexes. Both pesticides also altered steady state mRNA expression levels of a set of genes chosen to represent three possible mechanisms of endocrine disruption: (1) direct interaction with soluble sex steroid receptors, (2) biosynthesis of endogenous sex hormones, and (3) metabolism of endogenous hormones. *p,p'*-DDE acted as a weak estrogen, increasing the expression of vitellogenin and estrogen receptor α in the liver. *p,p'*-DDE also altered the expression of genes involved in the synthesis of endogenous hormones as well as their metabolism. Dieldrin, on the other hand, only altered expression of vitellogenin and not estrogen receptor α . Dieldrin also altered the expression of genes involved in hormone synthesis and metabolism, and it dramatically lowered plasma hormone levels. Both pesticides targeted expression of genes involved in all three modes of action, suggesting that they each have multiple modes of action.

Keywords

p,p'-DDE; Dieldrin; Largemouth bass; Quantitative real-time PCR; Endocrine disruptors; Organochlorine pesticides

1. Introduction

In vertebrates, normal reproduction is controlled by the hypothalamic-pituitary gonadal (HPG) axis through a cascade of hormones whose concentrations are tightly regulated by hormonal action and feedback control mechanisms. Any chemical that alters the concentration of endogenous hormones or mimics their action by binding or interfering with the binding of hormones to their natural receptors can disrupt this process. Transient or permanent disruption

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in normal reproductive function has been linked to chemicals that interrupt normal functioning of the endocrine system, but the mechanisms involved are still unclear (Jobling and Sumpter, 1993;Guillette et al., 1994;Gimeno et al., 1996;Arukwe, 2001;Hayes et al., 2002).

Even though most organochlorine pesticides (OCPs) have been banned for decades, they are still highly persistent in the environment. They were widely used in agriculture from the 1950s to the 1970s, and as one might expect they persist in sediments at the bottom of lakes and in the soils. Fish that are stocked into contaminated lakes fail to reproduce raising the possibility that exposure to OCPs has impaired their endocrine systems (Johnson, 1999). Our focus is to understand the molecular mechanisms underlying endocrine disruption of fish exposed to OCPs.

The three main pathways of endocrine disruption are: (1) interaction of endocrine-disrupting chemicals (EDCs) with the hormone receptors; (2) alteration of processes involved in steroid synthesis; (3) alteration of processes involved in sex steroid metabolism. Many EDCs interact with sex hormone receptors, such as estrogen receptors (ERs) or androgen receptor (AR) by acting as agonists or antagonists. Three different ER isotypes have been identified in fish (Hawkins et al., 2000;Legler et al., 2002;Sabo-Attwood et al., 2004;Filby and Tyler, 2005). They were originally named ER α , ER β and ER γ , but have recently been reclassified as ER α , ER β b and ER β a, respectively (Hawkins and Thomas, 2004). These receptors are expressed to different levels in endocrine active tissues and respond differently to 17 β -estradiol (E₂) concentrations at the message and activity levels (Sabo-Attwood et al., 2004). It is likely that they control different sets of genes.

Environmental contaminants may also disrupt the endocrine system by altering hormone homeostasis, which is maintained by tight control of hormone synthesis and metabolism. The first, and rate limiting, step in steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by StAR (steroidogenic acute regulatory protein) (Strauss et al., 1999;Christenson and Strauss, 2001;Stocco, 2001). Through a series of enzyme reactions, cholesterol is converted into testosterone (T), which can then be converted either to E₂ by the enzyme aromatase (CYP19) or to 11-ketotestosterone (11-KT) by the enzyme cytochrome P450 11 β -hydroxylase (CYP11 β). 11-KT is the major androgen in fish. The metabolism of steroids occurs by cytochrome P450 mediated reactions. CYP1A and CYP3A have been shown to metabolize endogenous steroids and detoxify drugs and environmental chemicals (Brian et al., 1990;Guengerich, 1991;Arukwe et al., 1997;Buhler and Wang-Buhler, 1998;Miranda et al., 1998;Sturm et al., 2001;Tseng et al., 2005). Bass have two isoforms of CYP3A (CYP3A68 and CYP3A69), which appear to differ in their activities and gene expression levels (Barber, D., personal communication). Alterations in these pathways could lead to changes in levels of endogenous steroids.

Largemouth bass (*Micropterus salmoides*, LMB) were chosen for this study, as they are known to be sensitive to environmental chemicals (Gross et al., 2000). The chosen OCPs were *p,p'*-DDE and dieldrin. *p,p'*-DDE is a product of the degradation of the pesticide DDT, as well as its most persistent metabolite. Dieldrin is considered as one of the most toxic pesticides, being included in the so-called “Dirty Dozen” from the Stockholm Convention on Persistent Organic Pollutants (POPs) together with its precursor, aldrin, and *p,p*-DDT. Dieldrin comes from the degradation of aldrin, although it was also released as a pesticide itself. It has been related to neurodegenerative disorders (Kitazawa et al., 2001,2003;Uversky et al., 2001;Kanthasamy et al., 2003,2005). *p,p'*-DDE and dieldrin were selected for this study because they are present at high levels in Florida lakes, where impaired endocrine and reproductive functions have been found in LMB (Johnson, 1999) and American alligators (*Alligator mississippiensis*) (Guillette et al., 1994,1995,1999;Marburger et al., 2002).

The main objective of this study was to begin to elucidate the pathways by which long-term exposure to *p,p'*-DDE and dieldrin cause endocrine disruption in LMB, in particular to understand how steroid hormone plasma levels are altered. We examined the expression levels of target genes that are involved in hormone homeostasis as well as genes that are targeted specifically by the ER signaling pathway.

2. Materials and methods

2.1. Animals and sample collection

Adult LMB (>2 years old) were purchased from American Sport Fish Hatchery (Montgomery, AL) in October 2003. Fish were maintained at the United States Geological Survey, Biological Resource Division, Center for Aquatic Resource Studies (USGS-BRD-CARD) facility in Gainesville, FL, in 6000 l concrete raceways, equipped with a flow-through system supplied by on-site well water and aeration. LMB were fed chemically treated floating pellets 5 days per week for 120 days beginning 4th November 2003. Sex ratio was unknown at the beginning of the study because gender cannot be determined non-invasively this early in the reproductive cycle. 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) and 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene (dieldrin) were incorporated into floating pellet fish feed by Zeigler Brothers, Inc. (Gardners, PA). Samples of each feed type and dose were tested for chlorinated pesticides by New Jersey Feed Laboratory, Inc. (Trenton, NJ). Feed concentrations were 5.3 and 45.9 µg/g for *p,p'*-DDE (low and high dose, respectively) and 0.4 and 0.81 µg/g for dieldrin (low and high dose, respectively). Samples were collected (6 females and 6 males from each treatment, and three control samples for each sex) after 120 days of exposure. Fish were euthanized by submersion in a water bath containing 50–110 ppm tricaine methanesulfonate (MS-222) and sacrificed by a sharp blow to the head followed by cervical transection in accordance with the University of Florida's Institutional Animal Care and Use Committee (IACUC) guidelines. Blood was collected from the caudal vein for plasma E₂ and 11-KT measurements, which were performed using a radioimmunoassay (RIA) as previously described (Sepúlveda et al., 2001). Liver and gonadal tissues were excised, immediately flash frozen in liquid nitrogen, and stored at –80 °C until RNA was isolated. Carcasses were extracted and analyzed by gas chromatography–mass spectrometry (GC–MS) for measurement of *p,p'*-DDE and dieldrin concentrations, using the method reported in Gelsleichter et al. (2005) and described in detail in Muller et al. (2004). The analyses were performed in the Analytical Toxicology Core Laboratory (University of Florida).

2.2. RNA extraction

Total RNA was isolated from LMB hepatic and gonadal tissue with the RNA Stat-60 reagent (Tel-test, Friendswood, TX). Briefly, 30–50 mg of tissue was homogenized in Stat-60, chloroform was added, and the mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C. The extraction process was repeated and the pooled RNA was precipitated in isopropanol. Following centrifugation, the pellet was washed with 70% ethanol, air dried, and resuspended in an appropriate volume (50–150 µl) of RNA secure (Ambion, Austin, TX) to inactivate RNases following the manufacturer's protocol. A total of 10 µg of RNA was treated with DNase to avoid contaminating DNA using DNA-free (Ambion, Austin, TX) following the manufacturer's protocol. The quantity and quality of total RNA were assessed by spectrophotometric readings at 260 and 280 nm (260/280 ratios around 2.0), and by electrophoresis through a 1% formaldehyde agarose gel stained with ethidium bromide, as previously described (Bowman et al., 2002).

2.3. Real-time PCR

Total RNA (1 µg) was reverse transcribed into cDNA using 3 µl random primers (0.1 µg/µl), 0.8 µl dNTP mix (25 mM each dNTP), 2 µl transcription buffer (10×), 1 µl StrataScript RT (50 U/µl), and 0.5 µl RNase Block (40 U/µl) in a final volume of 20 µl (all reverse transcription reagents were from Stratagene, La Jolla, CA). The resulting cDNA was used as a template for real-time PCR reactions. The genes analyzed for each sample were: vitellogenin (vtg), estrogen receptors (ER α , ER β b, ER β a) (Sabo-Attwood et al., 2004), androgen receptor (AR), cytochrome P450 3A68 (CYP3A68) and 3A69 (CYP3A69), and cytochrome P450 1A (CYP1A) in liver; steroidogenic acute regulatory protein (StAR), gonadal aromatase (CYP19), ER α , ER β b, ER β a and AR in gonad. Specific primers to each gene were designed to perform real-time PCR (Table 1).

Each real-time PCR reaction consisted of 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 62.5 µg of the primers and 1 µl of cDNA in a 25 µl reaction. The PCR conditions were 95 °C for 3 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA). The real-time PCR results for the samples treated with *p,p'*-DDE and dieldrin were normalized to 18S rRNA (Applied Biosystems, Foster City, CA) and analyzed using the $\Delta\Delta C_t$ method. Differences between the expression levels for the different pesticides, treatments, tissues and gender were analyzed statistically by multiple regression using Sigma STAT software (Jandel, CA) with a level of significance: $p \leq 0.05$. Significant differences ($p \leq 0.5$) between control and treated samples were determined by Student's *t*-test.

3. Results

3.1. Body burdens from dietary exposure

Chemical analyses to detect *p,p'*-DDE and dieldrin were performed on the whole fish carcass (6 females and 6 males from each treatment, and three control samples for each sex). Recovery ranged from 65 to 100% (detection limit of 0.75–1.5 ng/g). The total *p,p'*-DDE concentration found in fish treated with *p,p'*-DDE was 1.003 ± 0.641 µg/g body weight for the lower dose (measured dietary dose of 5.3 µg/g feed) and 7.912 ± 1.593 µg/g body weight for the higher dose (measured dietary dose of 45.9 µg/g feed). For dieldrin, body burden amounts were 0.204 ± 0.038 µg/g body weight for the lower dose (measured dietary dose of 0.4 µg/g feed) and 0.265 ± 0.050 µg/g body weight for the higher dose (measured dietary dose of 0.81 µg/g feed).

3.2. Measurement of endogenous hormones

One useful indicator of endocrine disruption is the ratio of female to male plasma sex hormone concentrations, E₂/T or E₂/11-KT (Folmar et al., 1996,2001;Lavado et al., 2004). During the reproductive phase, female fish that are in the process of oogenesis normally have high E₂/T ratios (above 1), whereas males normally have low E₂/T ratios (below 1). In fish from contaminated sites, the E₂/T ratios of females and males often overlap, resulting in indistinguishable profiles (Johnson, 1999). The overlapping ratios suggest that alterations occur with hormone homeostasis, either due to impaired synthesis and/or metabolism.

The concentrations of plasma E₂ and 11-KT for control fish, fish exposed to *p,p'*-DDE and fish exposed to dieldrin are plotted in Fig. 1 (all doses combined). The results show that long-term exposure to either of the two chemicals causes alterations of the E₂/T ratios such that there is little distinction between females and males. For fish treated with *p,p'*-DDE, there was an overall increase in 11-KT levels for both sexes and a decrease in E₂ levels for females. For dieldrin, the overall hormone levels decreased in both sexes. The overlapping pattern of hormone ratios for females and males in both treatments is consistent with the typical pattern found in fish from contaminated sites.

3.3. Expression of selected genes in liver and gonad from fish exposed to *p,p'*-DDE and dieldrin

Using the primer sets described above, we used real time PCR to measure the expression levels of *vtg*, *ERα*, *ERβa*, *ERβb*, *AR*, *CYP1A*, *CYP3A68* and *CYP3A69* in liver, and the expression of *StAR*, *ERα*, *ERβa*, *ERβb*, *AR* and *CYP19* in gonads. The results were plotted as the log 2 of the fold induction over controls calculated using the $\Delta\Delta C_t$ method. With this logarithmic transformation, scale increases and bars of the same height depict decreases of the same magnitude. Only log 2 results higher than 0.58 or lower than -0.58 were considered as important, as they would correspond to 1.5-fold increase or decrease using the $\Delta\Delta C_t$ method. The results were separated into different graphs (Figs. 2–7) representing the three different ways of action of endocrine disruptors: (a) receptor-related, (b) hormone synthesis and (c) hormone metabolism, as explained below.

3.3.1. Receptor-related

Genes that report on receptor related activities were *ERs α*, *βb* and *βa*, *AR* and *vtg*. Exposure to *p,p'*-DDE up regulated expression of *vtg* and *ERα* in the livers of both sexes, in a dose-dependent manner. *ERα* increased by 32-fold in females and 16-fold in males (both at the higher dose), suggesting that *p,p'*-DDE is estrogenic. *p,p'*-DDE exposure had the opposite effect on *ERβb* and *ERβa* expression in livers where both were decreased in males and females. *ERβa*, decreased by 16-fold in females and 32-fold in males. *p,p'*-DDE exposure also decreased the levels of *AR* mRNA in the livers of females, but not in males (Fig. 2). In the ovaries, *ERα*, *ERβb* and *AR* were down regulated and no significant changes were detected for *ERβa*. In males *AR* was down regulated, but *ERβb* and *ERβa* were up regulated, and no significant changes were detected for *ERα* (Fig. 2).

The low dose of dieldrin up regulated the expression of *vtg* in the livers of both sexes (up to four-fold in females, but only by two-fold in males), but the high dose was only effective in females. Surprisingly, dieldrin did not also increase *ERα* expression, a gene that is thought to be more sensitive to ER-mediated responses than *vtg* (Larkin et al., 2003). Mixed effects were observed on expression levels of *ERβb* and *AR* in the liver. *ERβb* was down regulated by the lower dose and up regulated by the higher dose; *ERβa* was down regulated only at the lower dose; *AR* was up regulated at the lower dose but only in females. In the gonad, dieldrin exposure down regulated *ERβb* and *ERβa* at the lower dose for both sexes; *ERα* and *AR* were down regulated in males; *AR* was up regulated in females (Fig. 3).

3.3.2. Hormone synthesis

Exposure to *p,p'*-DDE up regulated *StAR* expression in male and female gonads, markedly so in males, where mRNA levels increased 16-fold at the high dose. *p,p'*-DDE exposure decreased *CYP19* expression in the gonads by eight-fold in females and by four-fold in males (Fig. 4). Dieldrin exposure also increased *StAR* mRNA levels in the gonads of both sexes, with greater changes observed in the ovary (up to four-fold) than in the testis (two-fold). *CYP19* was up regulated in females by three-fold and down regulated in males by two-fold (Fig. 5).

3.3.3. Hormone metabolism

Exposure to *p,p'*-DDE increased *CYP3A68* and *CYP3A69* expression in the liver of both sexes. In females, both enzymes went up by two-fold. In males, *CYP3A68* went up by four-fold, while *CYP3A69* went up by two-fold. In contrast, the lowest concentration down regulated *CYP1A* by eight-fold for both females and males (Fig. 6).

Dieldrin had opposite effects on the two *CYP3A* isoforms. *CYP3A68* was up regulated only in females by two-fold, while *CYP3A69* was down regulated by three-fold (higher

concentration). In males the lower concentration of dieldrin up regulated CYP3A69 by two-fold. CYP1A was down regulated by 16-fold for the lower dose in both sexes (Fig. 7).

3.4. Comparison of the gene expression patterns in samples treated with *p,p'*-DDE and dieldrin

A summary of the differences observed in gene expression changes caused by the two chemicals is presented in Fig. 8. The doses chosen are the higher dose for *p,p'*-DDE and the lower dose for dieldrin, as these are the ones with the most changes. In order to compare patterns of gene expression changes in all the treatments, genes were arranged from the most up regulated to the most down regulated based on the results for *p,p'*-DDE for both liver and gonad. All data is plotted as the $\log_2$ of the $\Delta\Delta Ct$ (Fig. 8).

In the liver, the pattern for males treated with *p,p'*-DDE was very similar to that for females, where ER α , vtg, CYP3A68 and CYP3A69 were up regulated, and ER β b and ER β a were down regulated (Fig. 8A and B) except for AR, which did not change in males but was down regulated in females. In the gonad, more differences between the two sexes were observed. While every gene except StAR was down regulated in females, only CYP19 and AR were down regulated in males (Fig. 8A and B).

The overall expression patterns induced by dieldrin in females and males were very different from the patterns induced by *p,p'*-DDE. Both females and males had similar directional changes in their patterns of gene expression for both tissues, but the extent of change varied somewhat for the tested genes. The main differences compared to *p,p'*-DDE for the liver were that ER α was not up regulated by dieldrin, and CYP1A was highly down regulated in both sexes. In the gonad, both ER β a and ER β b were highly down regulated by dieldrin in both sexes, whereas StAR, as in *p,p'*-DDE, was up regulated (Fig. 8C and D).

4. Discussion

OCPs are bioaccumulated into higher organisms, largely through dietary intake. To mimic this route of exposure, LMB were exposed to *p,p'*-DDE and dieldrin by incorporating these compounds into their food. The exposure paradigm used in this study produced body burdens of OCPs comparable to those observed in muck farms and contaminated lakes in Central Florida (Marburger et al., 2002). Largemouth bass living in these areas presented levels of dieldrin in fat of 0.5 ppm and concentrations of DDT of 13 ppm, while the DDT concentration in the sediments was much lower than those for *p,p'*-DDE (0.06–0.6 ppm) (Marburger et al., 2002). Other studies have reported *p,p'*-DDE concentrations of 1.5 ppm in fish from the Adriatic Sea (Storelli et al., 2004) and 0.5 ppm in fish from Portland Harbor (Sethajintanin et al., 2004) indicating that the concentrations obtained in this study are environmentally relevant. We focused on *p,p'*-DDE and dieldrin because these are relatively stable metabolites of the parent compounds *p,p'*-DDT and aldrin.

Dietary treatment of LMB with *p,p'*-DDE led to an overall increase in plasma 11-KT levels in both males and females, and a decrease of E₂ only in females. Dieldrin treatment, on the other hand, led to a decrease of both hormones in males and females. Previously, dietary treatment of LMB with *p,p'*-DDE or dieldrin for 50 days resulted in decreases in plasma levels of E₂ and 11-KT, suggesting that duration of exposure may be an important parameter (Muller et al., 2004).

Similar to fish collected from the muck farms, treatment of LMB in the present study resulted in dramatically altered ratios of E₂/11-KT, such that it was difficult to separate males and females (Fig. 1). The sex hormone ratio (E₂/11-KT) has been used as an indicator of endocrine disruption because a specific estrogen to androgen ratio is necessary for sexual differentiation

and gamete development and maturation. Alterations of the ratio can result in incomplete or improper gonadal development (Folmar et al., 1996; Folmar et al., 2001; Lavado et al., 2004).

The ability of *p,p'*-DDE and dieldrin to alter endogenous hormone levels (Fig. 1) suggests that these compounds disrupt hormone homeostasis. While the mechanisms underlying these changes are complex, alterations in expression of genes involved in both synthesis and metabolism of endogenous hormones provide some insight. However, both compounds have also been described as weak estrogens and weak androgens by a variety of in vivo and in vitro assays, thus also implicating receptor-mediated modes of action (Kelce et al., 1995, 1997; Soto et al., 1994, 1995; Ramamoorthy et al., 1997; Arcaro et al., 1998; Sohoni and Sumpter, 1998; Maness et al., 1998; Andersen et al., 2002; Scippo et al., 2004).

4.1. Receptor-mediated mode of action

In the present study, exposure to *p,p'*-DDE increased the expression of ER α and vtg in the liver of both sexes. Increased expression of these genes occurs through the ER-mediated signaling pathway (Denslow et al., 1999; Sabo-Attwood et al., 2004). The activation is likely to have occurred by direct interaction with an ER, since plasma levels of E₂ were decreased by the treatment (Fig. 1). In the gonad, the effects on the ERs β b and β a varied by sex. In females, ER β b was down regulated and ER β a remained unchanged, while in males they were both up regulated in a dose responsive manner. ER β b and ER β a were down-regulated in the liver in both sexes, a finding that is different from the effects of E₂ or other estrogen like contaminants, such as nonylphenol, where the expression of these genes is not altered (Sabo-Attwood et al., 2004). So, although *p,p'*-DDE has an estrogenic effect, affecting vtg and ER α , it is acting differently from other estrogenic contaminants. AR was down regulated in the gonad of both sexes and in female hepatic tissue. *p,p'*-DDE is considered a competitive antagonist of AR androgen binding, inhibiting the transcription of androgen-regulated genes (Kelce et al., 1997). The binding of *p,p'*-DDE to the AR could increase the levels of androgen in plasma by displacement or by up regulation of steroid synthesis, as described below. Excess androgen in plasma could lead to a decrease in AR expression, as it has been previously shown in human blood cells (Khetawat et al., 2000).

The hepatic expression results differ from microarray experiments with LMB that were dosed with a single IP injection of *p,p'*-DDE (Larkin et al., 2002, 2003). In the microarray studies vtg mRNA levels increased only in males and decreased in females. Microarray experiments are known to be more qualitative than quantitative and much less sensitive than Q-PCR. But, the major difference between the two studies was the route and time of exposure, suggesting that some changes in gene expression depend on body burdens acquired over time and on the precise timing of the exposure during the reproductive season.

Dieldrin has been described as a weak estrogen (Soto et al., 1994; Ramamoorthy et al., 1997; Arcaro et al., 1998) and has also been reported to have weak anti-androgenic activity (Andersen et al., 2002). In this study, dieldrin acted as a very weak estrogenic compound, but only in females where vitellogenin was up regulated. There were no significant changes in ER α in the female or male liver, and whereas, vtg increased slightly at low dieldrin concentration and decreased slightly at high dieldrin concentration in males. Typically, ER α is the more sensitive gene for estrogens. The fact that it was not up regulated in females suggests that dieldrin may be acting through a different pathway than E₂. Nevertheless, dieldrin has been shown to activate the estrogen pathway in male rats (Okoumassoun et al., 2002).

4.2. Steroid biosynthesis

Expression of StAR, the rate limiting step in steroid synthesis, was increased in the gonads of both sexes following *p,p'*-DDE and dieldrin exposure. Regulation of StAR expression is

complex and is known to be affected by TGF β , TNF, GH, E₂, T, AR and xenobiotic compounds (Townson et al., 1996; Kanzaki and Morris, 1999; Attia et al., 2000; Walsh and Stocco, 2000; Morales et al., 2003; Houk et al., 2004). For *p,p'*-DDE, increased StAR expression is consistent with the elevated 11-KT levels in both sexes. Previous studies have demonstrated that StAR expression in testis is modulated by androgen receptor and increased expression of StAR occurs when AR activity is down regulated (Houk et al., 2004). *p,p'*-DDE is described as an anti-androgen and may be acting directly as an AR antagonist to increase gonadal StAR expression. However, with dieldrin treatment, plasma 11-KT levels declined in both sexes, contrary to what would have been expected with increased StAR mRNA expression. This observation suggests that alternative pathways are involved, possibly at the level of the hypothalamus and pituitary.

Expression of CYP19 (aromatase), the determining step in the conversion of T to E₂, was decreased in the gonads of both sexes following *p,p'*-DDE treatment. The magnitude of decrease in CYP19 expression was greater in females (eight-fold) than in males (four-fold). This decrease is consistent with the lowering of plasma E₂ in females, but not in males, where plasma E₂ remained constant. Nevertheless, 11-KT levels were highly increased in males, suggesting that overall steroidogenesis was increased in males. This could result in increased T, which was aromatized to E₂, maintaining this hormone level constant.

Dieldrin treatment, on the other hand, increased CYP19 expression in the gonads of females but decreased it in males. Plasma E₂ levels decreased in both sexes. This discordance between E₂ and CYP19 levels in females treated with dieldrin is confusing and may be due to compensatory changes in the hypothalamus and pituitary. Low plasma E₂ levels could feed-back to increase release of GnRH in an effort by the brain to achieve sex steroid homeostasis. If this occurs, it would also upregulate StAR.

4.3. Metabolism

The effects on the expression of the two CYP3A isoforms (CYP3A68 and CYP3A69) were quite different for the two treatments. *p,p'*-DDE exposure increased the expression of both isoforms in male and female livers. In the case of dieldrin, only the high dose affected the CYP3A isoforms in females, where CYP3A68 was increased and CYP3A69 was decreased. Both *p,p'*-DDE (Wyde et al., 2003) and dieldrin (Coumoul et al., 2001, 2002) can act directly as ligands for PXR, increasing CYP3A expression in human cell culture and rat. Homologs of the mammalian PXR have been found in several fish species and it is likely that they are present in LMB (Bresolin et al., 2005; Maglich et al., 2003).

CYP1A expression was most decreased at the lower concentrations of *p,p'*-DDE and dieldrin. Decrease of CYP1A expression by organochlorine pesticides has been shown in human cells as a result of either an ER-dependent effect or a PXR-related effect (Coumoul et al., 2001, 2002).

The patterns of action of the two pesticides were different, affecting the analyzed genes in distinct manners (Fig. 8). For each pesticide, females and males reacted similarly in the liver, but we observed clear sex-dependent differences in the gonads, as expected.

The present study suggests that exposure to these compounds changes the expression of genes critical in maintaining hormone homeostasis as well as genes directly linked to sex steroid hormone receptor-mediated pathways.

5. Conclusion

In conclusion, *p,p'*-DDE altered expression of genes involved in the three different pathways of endocrine disruption. *p,p'*-DDE acted as a weak estrogen and inhibited the expression of the AR, and altered genes involved in the synthesis of endogenous hormones as well as their metabolism. Dieldrin acted mainly to down-regulate the expression of mRNA for some of the hormone receptors, though it appeared not to act as an estrogen. Dieldrin also altered mRNAs for proteins involved in hormone synthesis and metabolism, and its effect on plasma hormone levels was quite dramatic, especially on E₂. However, it is not clear which changes are caused by the chemicals and which are compensatory responses driven by regulatory input from the HPG axis. Changes in gene expression patterns of this set of genes partially explained the observed changes in hormone levels. Nevertheless, we need more information on the endocrine pathways involved. Experiments with microarrays are in progress to determine the different pathways involved in a more global way.

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Fig 1.

Measurements of hormone levels in largemouth bass plasma from males and females performed using a radioimmunoassay (RIA). The graphs show the concentration of E₂ and 11-KT in plasma for control fish, fish treated with *p,p'*-DDE (both doses) and fish treated with dieldrin (both doses). All values are shown as pg/ml hormone in plasma.

Fig 2.

Changes in gene expression for the *p,p'*-DDE treatment for genes in the receptor-related pathway in liver and gonad for females and males. The treatments are low (5.3 μg/g feed, white bars) and high (45.9 μg/g feed, gray bars) *p,p'*-DDE. The following genes were analyzed for the liver: vtg, ERα, ERβb, ERβa and AR; and for the gonad: ERα, ERβb, ERβa and AR. Results are plotted as log₂ ΔΔCt. Statistical significance ($p = 0.05$) between control and treated fish and among treatments are represented with letters (a, b). Highlighted area corresponds to log₂ between 0.58 and -0.58, corresponding to a 1.5-fold change in either direction, the limits for up and down regulation, respectively.

Dieldrin: Receptor-Related

1. Liver

2. Gonad

Fig 3.

Changes in gene expression for the dieldrin treatment for genes in the receptor-related pathway in liver and gonad for females and males, as described in Fig. 2. The treatments are low (0.4 µg/g feed, white bars), and high (0.81 µg/g feed, gray bars) dieldrin.

p,p'-DDE: Hormone Synthesis (gonad)

Fig 4.

Changes in gene expression for the *p,p'*-DDE treatment in gonads for the hormone synthesis pathway. StAR and CYP19 were measured for females and males as described for Fig. 2.

Dieldrin: Hormone Synthesis (gonad)

Fig 5.
Changes in gene expression for the dieldrin treatment in gonads for the hormone synthesis pathway. StAR and CYP19 were measured for females and males as described for Fig. 3.

***p,p'*-DDE: Hormone Metabolism (liver)** **Fig 6.**

Changes in gene expression in the liver for the *p,p'*-DDE treatment for the hormone metabolism pathway. CYP3A68, CYP3A69 and CYP1A were measured for females and males as described for Fig. 2. down regulated in both sexes. In the gonad, both ERβa and ERβb were highly down regulated by dieldrin in both sexes, whereas StAR, as in *p,p'*-DDE, was up regulated (Fig. 8C and D).

Dieldrin: Hormone Metabolism (liver)

Fig 7.

Changes in gene expression in the liver for the dieldrin treatment for the hormone metabolism pathway. CYP3A68, CYP3A69 and Cyp1A were measured for females and males treated as described for Fig. 3.

Fig 8.

Summary of gene expression patterns for *p,p'*-DDE and dieldrin. Data for the high dose of *p,p'*-DDE and the low dose of dieldrin from Figs. 2–7 was re-plotted in the same order as for females treated with *p,p'*-DDE, where the genes were arranged from the most up regulated to the most down regulated, described as weak estrogens and weak androgens by a variety of *in vivo* and *in vitro* assays, thus also implicating receptor-mediated modes of action (Kelce et al., 1995,1997;Soto et al., 1994,1995;Ramamoorthy et al., 1997;Arcaro et al., 1998;Sohoni and Sumpter, 1998;Maness et al., 1998;Andersen et al., 2002;Scippo et al., 2004).

Table 1
Nucleotide sequences for the primers used for Q-PCR (5'–3' direction)

Gene	Forward	Reverse
Vtg	AAGCCCATCCAGGATCTC	GCTGCAGTGCCATGTATG
ER α	CGACGTGCTGGAACCAATGACAGAG	ACCTCCGGTCACTGATGATTTTCCTCCT
ER β b	CCGACACCGCCGTGGTGGACTC	AGCGGGGCAAGGGGAGCCTCAA
ER β a	GTGACCCGTCTGTCCACACA	TCTGGGGTCAGTGCAGGAGA
AR	CACCACAGAGAATGTGCCTGA	CAGGTGAGTGCGCCGTAA
CYP3A68	TGCACCGGGACCCTGAT	TGCTGAACCTCTCAGGTTTGAA
CYP3A69	GGCGAGCTGTGCATTCACT	CGTGGGTGATGAGAGGACTTG
CYP1A	GCAGCGCCGCTTTCC	CTGATGGCACTGAACTCAACAAGT
StAR	ACCCCTCTGCTGAGGCATTT	GGGCTCCACCTGCTTCTTG
CYP19	TGGATCAAGTGGATGTCCTCAGT	CCAGGAAGAGTCTGTTGGAGATG