

# Up-regulation of the alligator CYP3A77 gene by toxaphene and dexamethasone and its short term effect on plasma testosterone concentrations

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## Abstract

In this study we describe an alligator hepatic CYP3A gene, CYP3A77, which is inducible by dexamethasone and toxaphene. CYP3A plays a broad role in biotransforming both exogenous compounds and endogenous hormones such as testosterone and estradiol. Alligators collected from sites in Florida that are contaminated with organochlorine compounds exhibit differences in sex steroid concentrations. Many organochlorine compounds induce CYP3A expression in other vertebrates; hence, CYP3A induction by organochlorine contaminants could increase biotransformation and clearance of sex steroids by CYP3A and provide a plausible mechanism for the lowering of endogenous sex steroid concentrations in alligator plasma. We used real time PCR to examine whether known and suspected CYP3A inducers (dexamethasone, metyrapone, rifampicin, and toxaphene) up-regulate steady state levels of hepatic CYP3A77 transcript to determine if induction patterns in female juvenile alligators are similar to those reported in other vertebrates and whether toxaphene, an organochlorine compound found in high concentrations in Lake Apopka alligators, induces this gene. Estrogen receptor  $\alpha$  (ER $\alpha$ ), estrogen receptor  $\beta$  (ER $\beta$ ), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), and steroid-xenobiotic receptor (SXR) transcripts were also measured to determine whether any of these nuclear receptors are also regulated by these compounds in alligators.

Dexamethasone (4.2-fold) and toxaphene (3.5-fold) significantly induced CYP3A77 gene transcript, whereas rifampicin (2.8-fold) and metyrapone (2.1-fold) up-regulated ER $\beta$  after 24 h. None of the compounds significantly up-regulated AR, ER $\alpha$ , GR, PR, or SXR over this time period. Plasma testosterone (T) did not change significantly after 24 h in alligators from any of the treatment groups. Dexamethasone treated animals exhibited a strong relationship between the 24 h plasma T concentrations and CYP3A77 ( $R^2 = 0.9$ , positive) and SXR ( $R^2 = 0.77$ , negative) transcripts, which suggests that the expression of these genes is related to plasma T in alligators.

In light of our findings, we hypothesized that higher steady state CYP3A77 (and possibly SXR) gene expression would be observed in alligators collected from Lake Apopka, a polluted lake containing organochlorine compounds known to induce CYP3A isoforms in other taxa. Therefore, we measured basal levels of CYP3A77 and SXR gene transcripts in wild juvenile alligators collected from Orange Lake (reference lake), Lake Woodruff (reference lake), and Lake Apopka (contaminated lake). We found that no differences existed in CYP3A77 or SXR gene expression among animals from the lakes sampled suggesting that exposure to organochlorine compounds at concentrations present in Lake Apopka does not lead to variation in the expression of these genes, although capture stress could be interfering with these results since the glucocorticoid dexamethasone induces CYP3A77 transcript in alligators.

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## 1. Introduction

Juvenile female alligators inhabiting the Belle Glade area located at the southern end of Lake Okeechobee (FL) exhibit lower plasma testosterone (T) and estradiol-17 $\beta$  (E $_2$ ) concen-

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trations than females inhabiting other less contaminated sites (Gunderson et al., 2004). Alterations in gonadal steroidogenesis, plasma sex steroid binding proteins, hypothalamus or pituitary function, and/or the hepatic biotransformation of steroids are among the proposed mechanisms through which plasma hormone homeostasis may be altered. Differences in hepatic total testosterone hydroxylase activity have been demonstrated among juvenile alligators inhabiting a reference site and contaminated sites in Florida with a sexually dimorphic pattern being observed in Lake Woodruff alligators (reference population) that is not observed in alligators from Lakes Apopka or Okeechobee (Gunderson et al., 2001). Xenobiotic exposure could activationally disrupt sexually dimorphic patterns by up-regulating hepatic enzymes and in doing so increase urinary clearance of T. In this study we identified an alligator hepatic cytochrome P-4503A gene (CYP3A77) that is responsible for biotransforming T in other taxa, investigated its inducibility by known inducers, examined the short term effect of up-regulating this gene on plasma testosterone (T) concentrations, and measured its basal expression in wild juvenile alligators inhabiting reference and contaminated sites in Florida.

The predominant hydroxylated T metabolite produced by alligator hepatic microsomes co-migrates with the mouse 6 $\beta$ -hydroxytestosterone metabolite on thin layer chromatography plates (Gunderson, 2005). The biotransformation of testosterone to 6 $\beta$ -hydroxytestosterone is generally associated with CYP3A activity (Gibson et al., 2002). CYP3A (or CYP3A like activity) has been described and/or characterized in rat, human, baboon, monkey, dog, chicken, frog, and crocodile (among other taxa), and a phenobarbital (PB) and 3-methylcholanthrene (3-MC) inducible cytochrome P-450 enzyme that cross-reacts with rabbit-anti-trout CYP3A antibody has been described in alligators (Ertl et al., 1998; Reilly et al., 1999; Ertl et al., 1999b; Ourlin et al., 2000). T, estradiol (E<sub>2</sub>), progesterone (P<sub>4</sub>), and androstenedione are among the substrates metabolized by CYP3A, and numerous chemicals and pesticides (DDT, dieldrin, mirex, chlordane, methoxychlor, endosulfan, trans-nonachlor, non-polar PCBs, nonylphenol, phthalic acids) induce CYP3A in humans and rodent models (Li et al., 1995; Nims and Lubet, 1995; Nims et al., 1998; Coumoul et al., 2002; Gibson et al., 2002). It is therefore conceivable that alligators inhabiting an environment contaminated with organochlorine pesticides known to induce CYP3A would exhibit elevated CYP3A activity leading to increased T clearance from the plasma. In mice, DDE and mirex exposure induces CYP3A (among other CYP isoforms) and increases hydroxylated T metabolite formation *in vitro* (2.5–3-fold) (Dai et al., 2001). In chickens, induction of hepatic cytochrome P-450 enzymes by phenobarbital, DDT, and PCBs is associated with decreases in plasma E<sub>2</sub> concentrations (Chen et al., 1993, 1994). In humans, phenobarbital increases urinary excretion of T by more than 60%, although it does not alter endogenous or exogenously administered T (Bammel et al., 1992).

Several nuclear receptors are known to mediate the up-regulation of CYP3A transcript (Gibson et al., 2002). Blumberg et al. (1998) first proposed the ‘steroid sensor hypothesis’ in

which a broad specificity nuclear receptor regulates the induction of enzymes responsible for the metabolism of endogenous and exogenous compounds. This hypothesis has been supported by subsequent studies that examined the relationship between steroid-xenobiotic receptor (SXR) (also referred to as PXR or PAR) and CYP3A as well as other enzymes (Gibson et al., 2002). Many of the compounds that induce CYP3A are also inducers of SXR (Gibson et al., 2001). Interestingly, organochlorine compounds induce CYP3A in mammalian models through PXR (SXR) mediated pathways (Sierra-Santoyo et al., 2000; Coumoul et al., 2002). Further more, the nuclear receptors SXR, glucocorticoid receptor (GR), RXR (retinoid X receptor), and possibly ER, androgen receptor (AR), and PR (progesterone receptor) appear to work together in the receptor dependent regulation of CYP3A in different taxa, and estrogens, androgens, corticosteroids, and progestins are all known to act as substrates and/or inducers of CYP3A and activators of SXR in different model systems (Huss et al., 1996; Wang and Strobel, 1997; Blumberg et al., 1998; Ertl et al., 1998; Reilly et al., 1999; Ertl et al., 1999b; Ourlin et al., 2000; Gibson et al., 2001; Cai et al., 2003; Anakk et al., 2003a). Potential SXR, ER, PR, and GR response elements have been identified on the proximal promoters of various CYP3A isoforms (Jounaidi et al., 1994; Huss et al., 1996; Blumberg et al., 1998; Gibson et al., 2001; Anakk et al., 2003a). In this study we also examined whether short term exposure to known CYP3A inducers and toxaphene up-regulates alligator nuclear receptor gene transcripts for which we had primers and gene sequences (AR, ER $\alpha$ , ER $\beta$ , GR, PR, SXR).

This study consists of two parts. In the first part we used real time quantitative PCR to examine the up-regulation of CYP3A77 and six nuclear receptors (AR, ER $\alpha$ , ER $\beta$ , GR, PR, and SXR) in captive raised female alligator livers by three known CYP3A inducers (dexamethasone, rifampicin, and metyrapone) and by toxaphene, an organochlorine pesticide found in high concentrations in Lake Apopka alligator eggs (Woodward et al., 2003). Our goals were to first of all determine whether known mammalian and chicken CYP3A inducers and the organochlorine pesticide toxaphene modulate CYP3A77 and nuclear receptor transcripts in yearling female alligators, and to examine whether changes in plasma T concentrations occur after 24 h of exposure to CYP3A inducers. The second part of the study examined steady state CYP3A77 and SXR expression in wild juvenile alligators collected from three lakes in Florida with varying degrees of contamination. Specifically, we asked whether CYP3A77 and SXR gene transcripts were expressed in wild juvenile alligators, and further determined if variation in the basal level expression of these genes is observed in juvenile alligators reared in Florida lakes with different amounts of contamination.

## 2. Methods

### 2.1. Up-regulation study

The first part of this study involved injecting captive raised female yearling alligators with chemicals that are known or sus-

pected to induce CYP3A in chickens and/or mammals. Dexamethasone and metyrapone both up-regulate CYP3A37 in chickens, with metyrapone being the stronger inducer (Ourlin et al., 2000). Rifampicin, a CYP3A inducer in human and rabbit hepatocytes, does not induce CYP3A in chickens or rats (Ourlin et al., 2000; Gibson et al., 2002). Toxaphene exhibits a strong positive correlation with 6 $\beta$ -testosterone hydroxylase activity in Harp seals, suggesting that it participates in the regulation of CYP3A in marine mammals (Wolkers et al., 2000). Furthermore, ketoconazole, a CYP3A inhibitor, caused a dose dependent inhibition of toxaphene metabolism in seal (van Hezik et al., 2001). Toxaphene is found in relatively high concentrations in alligator eggs collected from Lake Apopka (mean 9.4 ppm) relative to Lakes Orange (not detected) and Woodruff (mean 0.084 ppm) (Woodward et al., 2003).

We chose to focus on females in this study because a small number of males were available and we could not pool males and females based on a previous study that reported sex differences in the induction of CYP3A in rodent livers. DDT was demonstrated to increase CYP3A2 18-fold in female rats, but less than three-fold in male rats (Sierra-Santoyo et al., 2000). Furthermore, female juvenile alligators collected from the area surrounding Belle Glade on Lake Okeechobee exhibited depressed plasma T and E<sub>2</sub> when compared to those from other sites in south Florida (Gunderson et al., 2004). No differences in plasma T were observed among male juvenile alligators collected from the same sites in south Florida, suggesting that the homeostatic mechanisms regulating plasma steroid concentrations are more sensitive to contaminant exposure in female alligators.

Captive raised yearling female alligators from Lake Woodruff were injected, via the post-cranial vertebral vein, with vehicle (DMSO), 50  $\mu$ M dexamethasone (1.44 mg/kg), 1 mM metyrapone (16.5 mg/kg), 50  $\mu$ M rifampicin (3 mg/kg), 50  $\mu$ M toxaphene (1.6 mg/kg), or received a needle stick (sham). DMSO was purchased from Fisher Scientific (# BP231-1). Dexamethasone (Sigma # D1756), metyrapone (2-methyl-1,2-di(3-pyridyl-1-propanone)) (Sigma # 85,652-5), and rifampicin (Sigma # R3501) stock solutions were mixed in DMSO. Toxaphene (# F106AS), dissolved in acetone (2500  $\mu$ g/ml), was purchased from Chem Service Inc., West Chester, PA. A stock toxaphene solution was mixed by evaporating the acetone under a steady stream of dry air and re-suspending the toxaphene in DMSO. Stock concentrations were mixed so that the same volume of DMSO vehicle was injected per gram of body weight (0.13  $\mu$ l DMSO/gram). DMSO concentrations in the plasma were estimated to be ~0.2%.

The reported concentration for each compound is based on estimated circulating concentrations in plasma as described in (Edwards et al., 2004). Briefly, 10% of the alligator's body weight in grams was estimated to be the volume of blood in milliliters, of which 74% was estimated to be plasma volume. Each stock solution was mixed so that the same volume of DMSO/compound would be injected per gram body weight. Animals were euthanized via an overdose of sodium pentobarbital injected into the post-cranial vertebral vein 24 h ( $\pm$ 8 min) after injection of the test compounds. Liver tissue was removed and flash frozen in liquid nitrogen within 5 min of death (mean

2 min 50  $\pm$  47 s). Blood samples were collected immediately before injection and 24 h after the injection of the test compounds, just prior to the administration of sodium pentobarbital. Plasma T was measured using previously reported RIA techniques validated for alligators (Guillette et al., 1996, 1997; Gunderson et al., 2003, 2004).

We note that sample sizes differ throughout this study. Initially, five female alligators from Lake Woodruff were assigned to each treatment group but some of the liver samples could not be used to measure gene expression due to the mRNA being degraded during handling and processing. The plasma T samples from these animals were still usable, which explains the discrepancy in sample size for the plasma T concentrations reported in Fig. 5. Additional animals from unknown lakes in Florida, and raised under identical conditions as the Lake Woodruff animals used in this study, were treated at the same time and used to increase the sample size for the component of the experiment pertaining to the regression analyses examining the relationships between plasma T concentrations and the gene expression measured in this study. Patterns of up-regulation for these animals matched those for the animals from Lake Woodruff but we chose not to include them in the CYP3A and nuclear receptor analyses since we have data suggesting that a single exposure to contaminants during development permanently changes hepatic enzyme and nuclear receptor expression at later life stages (Gunderson, 2005). These animals were used in the analyses examining plasma T patterns over time and plasma T as it relates to mean relative gene expression since we were interested in how plasma T relates to hepatic CYP3A77 and nuclear receptor expression.

## 2.2. Field study

Sites examined in this study were chosen based on historical data and contaminant concentrations present in alligator eggs (or alligator serum) collected from the three lakes. Many of the organochlorine compounds are lipophilic and stored in the body fat of organisms exposed to this family of compounds. Maternal stores of these compounds can be transferred to eggs and serve as an indicator of contaminant concentrations present in the different lakes (Guillette et al., *in press*). Historically Lake Apopka has received runoff containing organochlorine compounds from extensive muck farming operations on its northern shoreline and a chemical spill in 1980 from the Tower Chemical Company located near (~1 mile) Gourd Neck Springs on the south shore of the lake (USEPA, 1994, 2004). Alligator eggs and alligator serum collected from alligators inhabiting Lake Woodruff and Orange Lake contain lower concentrations of organochlorine compound concentrations than eggs/animals inhabiting Lake Apopka (Guillette et al., 1999a,b,c; Woodward et al., 2003).

Five male and five female juvenile alligators (91–138.5 cm) were collected from Lakes Woodruff (April 16, 2002), Orange (April 17, 2002), and Apopka (April 18, 2002). Animals were hand grabbed using airboats on consecutive nights, at approximately the same time (21:00–01:00), to control for variation due to temperature, season, and diurnal cycles (Guillette et al., 1999a,b,c). The jaws were secured using rubber bands and the

animals placed in cloth bags for transport back to the University of Florida where necropsies were performed the same night. Animals were killed using an overdose of sodium pentobarbital injected into the post-cranial vertebral vein. Liver tissue was removed immediately after the animals were killed and flash frozen in liquid nitrogen for mRNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and quantitative real time polymerase chain reaction (qPCR).

2.3. RNA isolation, RT-PCR, and qRT-PCR

Isolation of RNA, RT-PCR, and qRT-PCR was conducted as described (Katsu et al., 2004). Total RNA was produced by using the TRIzol reagent (Invitrogen, Carlsbad, CA), and was treated with ribonuclease-free deoxyribonuclease-I (DNase I;

Invitrogen or Qiagen, Valencia, CA) to avoid contamination by genomic DNA. After the DNase I treatment, total RNA was purified using RNeasy total RNA isolation kit (Qiagen). The concentrations and quality of the RNA samples were estimated by measurement of optical density at 260 nm, checked by formaldehyde gel electrophoresis, and then the RNA was stored at -70 °C.

The cDNA was synthesized using the reverse transcriptase, Super Script II (Invitrogen) with random hexa-mer primer (Invitrogen) for the degenerate PCR, or with oligo(dT) adaptor primer (Takara, Otsu, Japan) for the 3'-rapid amplification of cDNA ends (RACE). One microgram of total RNA was reverse-transcribed in 20 µl of reaction mixture. Four microliters of cDNA was then used in 50 µl of the PCR reaction mixture with the template. The cDNA frag-

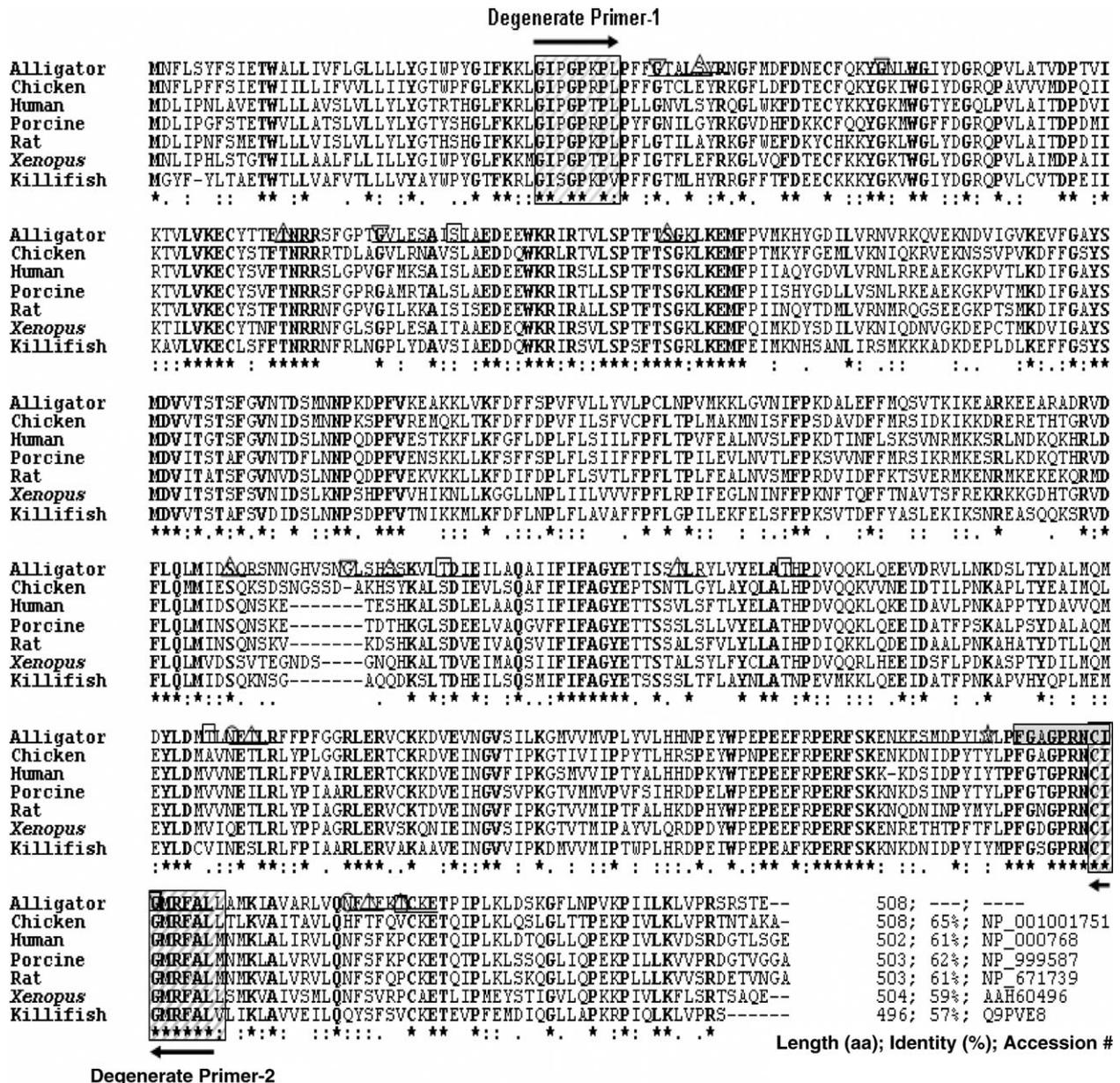


Fig. 1. Comparison of the deduced alligator CYP3A77 amino acid sequence with other species.

ment was amplified by using rTaq DNA polymerase (Takara) at an annealing temperature of 50 °C with the degenerate primers, 5'-GGGSATTCTGGGCCMARACWYTG-3' and 5'-GAGAGCAAAYCKCATSCCAATGCAG-3'. These primers were based on the conserved sequence for CYP3A in chicken (AJ250337) and mouse (NM\_177380) at the site as shown in Fig. 1. The 3'-end of this cDNA fragment was amplified using rTaq DNA polymerase (Takara) and the 3'-full RACE core set (Takara). First, the 3'-end cDNA was amplified with 3'-adaptor primer and 3'-RACE 1st primer, 5'-TGGATTTCCTGCAGCTGATG-3'. Sequentially, 4 µl of first PCR reaction was used in 50 µl of PCR as the template (2nd PCR). In the 2nd PCR, we used the 3'-adaptor primer and 3'-RACE 2nd primer, 5'-TGGAGTGAGCATTCTGAAAGGA-3'. The 3'-RACE primers were based on the sequence of the cDNA fragment obtained from the degenerate PCR as described above (or see AB186129). The PCR reaction using 3'-RACE primers were carried out at the annealing temperature of 59 °C.

The amplified cDNA fragments were purified by using the QIAquick Gel Extraction Kit (QIAGEN), and were ligated into the pGEM-T easy vector (Promega, Madison, WI). The sequencing reaction was carried out using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster, CA), and was analyzed on the ABI PRISM 377 or ABI PRISM 310 automatic sequencer (Applied Biosystems). The alligator CYP3A gene identified in this study (CYP3A77) was named by the cytochrome P450 nomenclature committee (Dr Nelson).

The quantitative real-time qPCR analyses were performed on an ABI PRISM 7000 or Gene Amp 5600 sequence detection system instrument using software with the default settings (Applied Biosystems, 1997). One microgram of DNase-treated total RNA was used in 20 µl of reverse transcription (RT) with super script II (Invitrogen) and random primer (Invitrogen). One microliter of RT product was used in 15 µl of PCR. The reactions

were carried out using SYBR Green PCR Master Mix (Applied Biosystems, 1997) with specific primer pairs (Table 1). The specificity of amplification was confirmed using the dissociation curve method. The mRNA expression of each target gene was normalized by an endogenous control, ribosomal protein L8, according to the comparative CT method (Applied Biosystems, 1997).

## 2.4. Statistical analysis

### 2.4.1. CYP3A77 up-regulation study

Relative gene expression for each treatment was calculated and statistically analyzed in relation to the DMSO (vehicle) treatment group (mean relative gene expression equal to one). Mean relative GR expression required no transformation to achieve homogeneity of variance using an homogeneity of variance *F*-test. Mean relative CYP3A77, SXR, and ERβ gene expressions were log transformed to achieve homogeneity of variance and a one-way ANOVA with Fisher's PLSD post hoc analysis was used to make comparisons among treatment groups. Homogeneity of variance could not be achieved using standard transformations for ERα and AR so a Kruskal–Wallis non-parametric analysis was used to determine differences among the sham, DMSO, and a specific treatment.

Simple linear regression was performed on log-transformed datasets to examine the relationship between CYP3A77 and each individual nuclear receptor measured in this study. Plasma T concentrations were log transformed to achieve homogeneity of variance. A paired *t*-test was performed to compare the initial and 24 h post-injection plasma T concentrations for each treatment. Simple linear regression analyses were performed on log transformed datasets to examine the relationship between the 24 h plasma T concentrations and mean relative CYP3A77, AR, ERα, ERβ, GR, PR, and SXR gene expressions.

Table 1  
The primers used for the quantitative real-time PCR

Name	Direction	Position (base)	Sequence (5' to 3')	Amplified length (base)	Gene Bank accession # or reference
Ribosomal protein L8	Forward	–	GGTGTGGCTATGAATCCTGT	–	Katsu et al. (2004)
	Reverse	–	ACGACGAGCAGCAATAAGAC		
CYP3A77	Forward	188	GCTTTGGTCCAACAGGTGTGT	79	AB186129
	Reverse	266	AGCACAGTGCGAATCCTCTTC		
SXR	Forward	417	GCCCCGATGTCTTCTCCATTCT	53	(Blumberg, unpublished data)
	Reverse	469	TCATGAAGGTGCTGAGATCCG		
GR	Forward	69	AAAAAACTGTCCCGCATGCC	104	AF525750
	Reverse	172	CGTTGGACTGCTGAATTCCTTT		
ERα	Forward	410	AAGCTGCCCTTCAACTTTTAA	72	AB115909
	Reverse	481	TGGACATCCTCTCCCTGCC		
ERβ	Forward	34	AAGACCAGGCGCAAAAGCT	72	AB115910
	Reverse	105	GCCACATTCATCATTCCAC		
PR	Forward	50	AAATCCGTAGGAAGAACTGTCCAG	72	AB115911
	Reverse	121	GACCTCCAAGACCATTCAC		
AR	Forward	205	TGTGTTTCAGGCCATGACAACA	103	AB186356
	Reverse	307	GCCCATTCACCATGCA		

#### 2.4.2. Field study

Mean relative CYP3A77 and SXR gene expression for each site was calculated relative to Lake Woodruff females (mean relative gene expression equal to one). No significant difference in body size existed among sites ( $p=0.71$ ) so body size was not used as a covariate in the analyses. Mean relative CYP3A expression values were log transformed to achieve homogeneity of variance, which was measured using an homogeneity of variance  $F$ -test. One-way ANOVA with Fisher's PLSD post hoc analysis was used for the comparison of CYP3A77 among lakes. Homogeneity of variance could not be achieved using standard transformations for SXR so Kruskal–Wallis non-parametric analysis was used for comparisons of mean relative SXR expression among sites. Simple linear regression was performed on log-transformed datasets to examine the relationship between mean relative CYP3A77 and SXR gene expressions.

Statview (SAS Institute, Cary NC) was utilized for all statistical analyses.

### 3. Results

#### 3.1. CYP3A77 and nuclear receptor up-regulation in female yearling alligators

The alligator CYP3A77 gene identified in this study is 65% homologous (deduced amino acid sequence) with the chicken CYP3A37 enzyme (Fig. 1). Dexamethasone (4.2-fold;  $p=0.01$ ) and toxaphene (3.5-fold;  $p=0.01$ ) induced CYP3A77 mRNA in yearling female alligators relative to DMSO injected animals (vehicle control). Metyrapone ( $p=0.18$ ) and rifampicin ( $p=0.78$ ) did not significantly induce CYP3A77. There was no vehicle (DMSO) effect on CYP3A77 gene transcription ( $p=0.91$ ) (Fig. 2).

Both metyrapone (2.1-fold;  $p=0.02$ ) and rifampicin (2.8-fold;  $p=0.005$ ) induced ER $\beta$  relative to DMSO injected animals, whereas dexamethasone ( $p=0.3$ ) and toxaphene ( $p=0.09$ ) did not elicit a significant effect (Fig. 3).

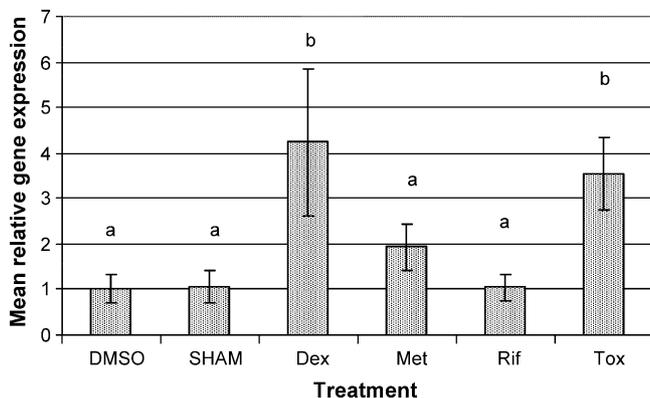


Fig. 2. Mean relative CYP3A77 gene expression (mean  $\pm$  S.E.) after 24 h in yearling female alligators injected with nothing (sham;  $N=5$ ), carrier (DMSO;  $N=4$ ), dexamethasone (Dex, 50  $\mu$ M;  $N=4$ ), metyrapone (Met, 1 mM;  $N=5$ ), rifampicin (Rif, 50  $\mu$ M;  $N=3$ ), and toxaphene (Tox, 50  $\mu$ M;  $N=5$ ). a,b denote a significant difference ( $p \leq 0.05$ ) between the treatments and the DMSO (vehicle) treated animals.

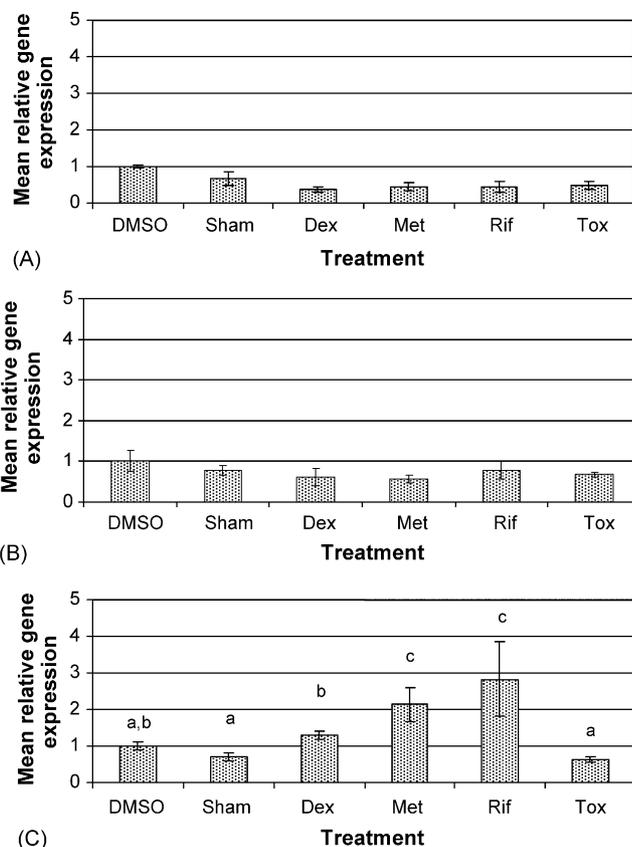


Fig. 3. Mean relative (A) AR, (B) ER $\alpha$ , and (C) ER $\beta$  gene expressions (mean  $\pm$  S.E.) after 24 h in yearling female alligators injected with nothing (sham;  $N=5$ ), carrier (DMSO;  $N=4$ ), dexamethasone (Dex, 50  $\mu$ M;  $N=4$ ), metyrapone (Met, 1 mM;  $N=5$ , rifampicin (Rif, 50  $\mu$ M;  $N=3$ ), and toxaphene (Tox, 50  $\mu$ M;  $N=5$ ). a,b denote significant differences ( $p \leq 0.05$ ) between the different treatment groups. A lack of superscripts mean that no significant differences were observed.

No significant changes ( $p > 0.05$ ) in SXR, GR, ER $\alpha$ , AR, or PR were observed among treatments relative to DMSO injected animals (Figs. 3 and 4).

Significant relationships were observed between CYP3A77 and ER $\alpha$  (metyrapone), ER $\beta$  (DMSO), and PR (metyrapone and toxaphene), although no clear pattern existed in relation to the dexamethasone treated group, which exhibited a significant up-regulation in CYP3A77 transcript (Table 2).

#### 3.2. Plasma T concentrations

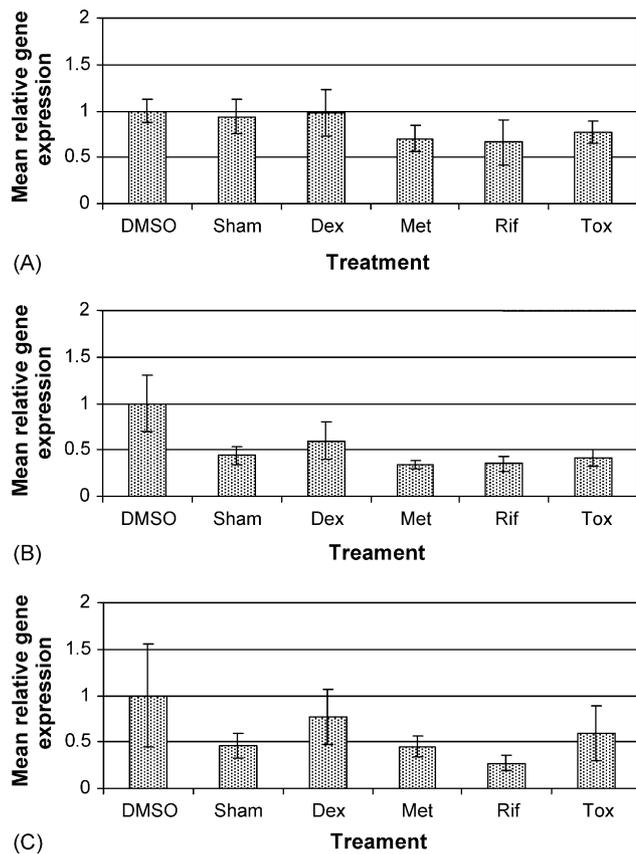
In this study, we were also interested in examining whether a relationship existed between CYP3A77 transcript and circulating plasma T concentrations since up-regulation of CYP3A by organochlorine pesticides could lead to increased clearance of plasma T. No change in plasma T concentrations was observed 24 h after the injections were administered in animals from any of the treatment groups ( $p > 0.2$ ) (Fig. 5).

Simple linear regression was used to evaluate whether relationships existed between 24 h plasma T concentrations and mean relative CYP3A77 and/or the nuclear receptor gene expressions measured in this study. Strong relationships existed between the 24 h plasma testosterone samples and mean rela-

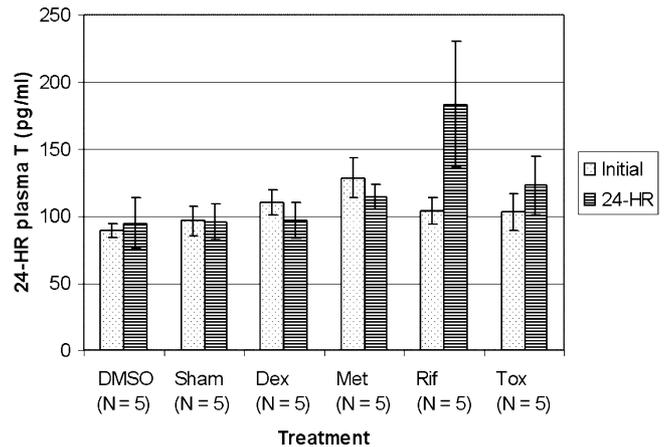
**Table 2**  
Simple linear regression examining the relationship between CYP3A77 and six nuclear receptors (AR, ER $\alpha$ , ER $\beta$ , GR, PR, SXR) in female yearling alligators injected with DMSO (carrier), dexamethasone (Dex) (50  $\mu$ M), metyrapone (Met) (1 mM), rifampicin (Rif) (50  $\mu$ M), toxaphene (Tox) (50  $\mu$ M), or a needle stick (sham) (Only significant relationships ( $p \leq 0.05$ ) are included)

Treatment	N	R <sup>2</sup>	p-value	Line equation
Grouped	26	0.16	0.04	Log(CYP3A77) = 0.72(log(ER $\alpha$ )) + 0.32
DMSO	4	0.96	0.02	Log(CYP3A77) = 3.25(log(ER $\beta$ )) - 0.06
Met	5	0.88	0.02	Log(CYP3A77) = 1.69(log(ER $\alpha$ )) + 0.66
		0.85	0.03	Log(CYP3A77) = 2.04(log(PR)) + 1.19
Tox	5	0.88	0.02	Log(CYP3A77) = -1.18(log(PR)) + 0.004

tive CYP3A77 (positive,  $R^2 = 0.9$ ,  $p = 0.01$ ) and SXR (negative,  $R^2 = 0.77$ ,  $p = 0.05$ ) gene expressions in animals from the dexamethasone treatment groups (Table 3). These animals also exhibited significant up-regulation of mean relative CYP3A77 gene expression, which suggests that compensatory mechanisms are possibly acting to maintain plasma T concentrations. A weaker negative relationship existed between 24 h plasma T concentrations and mean relative SXR gene expression when animals from all of the treatment groups were combined ( $R^2 = 0.28$ ,  $p = 0.001$ ) (Table 3). Other receptors exhibited patterns suggestive ( $p \leq 0.1$ ) of a relationship with plasma T. Specifically, mean relative AR expression exhibited a negative relationship with 24 h plasma



**Fig. 4.** Mean relative (A) GR, (B) PR, and (C) SXR gene expressions (mean  $\pm$  S.E.) after 24 h in yearling female alligators injected with nothing (sham;  $N = 5$ ), carrier (DMSO;  $N = 4$ ), dexamethasone (Dex, 50  $\mu$ M;  $N = 4$ ), metyrapone (Met, 1 mM;  $N = 5$ ), rifampicin (Rif, 50  $\mu$ M;  $N = 3$ ), and toxaphene (Tox, 50  $\mu$ M;  $N = 5$ ). There were no significant differences ( $p \leq 0.05$ ) between the different treatment groups.



**Fig. 5.** Plasma testosterone concentrations (mean  $\pm$  S.E.) measured prior to the first injection and 24 h post-injection in yearling female alligators injected with nothing (sham needle stick), carrier (DMSO), dexamethasone (Dex, 50  $\mu$ M), metyrapone (Met, 1 mM), rifampicin (Rif, 50  $\mu$ M), and toxaphene (Tox, 50  $\mu$ M). There were no differences ( $p \leq 0.05$ ) between initial and 24 h plasma T concentrations for any of the treatments.

T in sham animals ( $R^2 = 0.48$ ,  $p = 0.08$ ) (Table 3); mean relative ER $\beta$  gene expression exhibited a positive relationship with plasma T in rifampicin treated animals ( $R^2 = 0.65$ ,  $p = 0.1$ ) and a negative relationship with plasma T in toxaphene treated animals ( $R^2 = 0.45$ ,  $p = 0.1$ ) (Table 3); mean relative GR gene expression exhibited a negative relationship with 24 h plasma T concen-

**Table 3**  
Simple linear regression examining the relationship between testosterone in the 24 h plasma sample and mean relative CYP3A77, AR, ER $\alpha$ , ER $\beta$ , GR, PR, and SXR expressions

Treatment	N	R <sup>2</sup>	p-value	Equation
Grouped	35	0.14	0.03	Log(24 h T) = -0.27log(PR) + 2
		0.28	0.001	Log(24 h T) = -0.29log(SXR) + 2
Sham	7	0.48	0.08	Log(24 h T) = -0.43log(AR) + 1.9
		0.46	0.09	Log(24 h T) = -0.5log(ER $\beta$ ) + 1.9
Dex	5	0.9	0.01	Log(24 h T) = 0.4log(CYP3A77) + 1.7
		0.77	0.05	Log(24 h T) = -0.42log(SXR) + 2
Met	7	0.53	0.06	Log(24 h T) = -0.2log(SXR) + 2
Rif	5	0.65	0.1	Log(24 h T) = 0.9log(ER $\beta$ ) + 1.7
Tox	7	0.45	0.1	Log(24 h T) = -0.73log(ER $\beta$ ) + 2

Yearling alligators were injected with nothing (sham), carrier (DMSO), dexamethasone (Dex) (50  $\mu$ M), metyrapone (Met) (1 mM), rifampicin (Rif) (50  $\mu$ M), and toxaphene (Tox) (50  $\mu$ M). Only relationships suggesting significance are included based on either a  $p$ -value of  $\sim 0.1$  and/or  $R^2$ -values greater than 0.5.

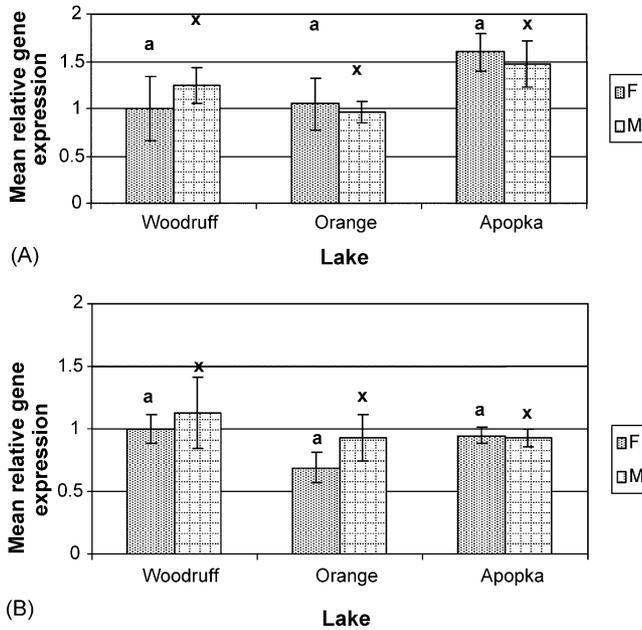


Fig. 6. Mean relative (A) CYP3A77 and (B) SXR gene expression (mean  $\pm$  S.E.) in juvenile alligators collected from lakes Woodruff, Orange, and Apopka.  $N=5$  for each group. Asterisk denotes a significant difference between males and females within each treatment ( $p \leq 0.05$ ); a,b denote significant differences among females from the different treatment groups; x,y denote significant differences among males from the different treatment groups.

trations in animals from the sham treatment group ( $R^2 = 0.46$ ,  $p = 0.09$ ) (Table 3); mean relative SXR gene expression exhibited a negative relationship with 24 h plasma T ( $R^2 = 0.53$ ,  $p = 0.06$ ) in metyrapone treated animals (Table 3).

### 3.3. Field study

#### 3.3.1. Mean relative CYP3A77 and SXR gene expression

Contrary to our predictions, basal levels of CYP3A77 mRNA were not sexually dimorphic in juvenile alligators (3–5-years old) from lakes Woodruff, Orange, or Apopka and mean relative CYP3A77 mRNA levels did not differ among sites ( $p > 0.05$ ). Similarly, SXR transcript levels did not exhibit sexual dimorphism in the same animals and no differences in mean relative gene expression were observed among the three sites ( $p > 0.05$ ) (Fig. 6).

Significant positive relationships between CYP3A77 and SXR transcripts were observed in animals from Orange Lake when males and females were grouped together ( $R^2 = 0.47$ ,  $p = 0.03$ ) and when females were considered separately ( $R^2 =$

0.76,  $p = 0.05$ ) (Table 4). A significant relationship was also observed when animals from the three lakes were grouped together ( $R^2 = 0.15$ ,  $p = 0.03$  grouped) and when females from these lakes were analyzed separately ( $R^2 = 0.3$ ,  $p = 0.03$ ). No significant relationships existed in animals from lakes Woodruff or Apopka (Table 4).

## 4. Discussion

This study was an initial step in the identification of the alligator CYP3A77 gene to determine whether its message can be measured in wild alligators and if known CYP3A inducers up-regulate its expression. We were interested in examining the regulation of CYP3A77 in alligators because of the role this enzyme plays in the metabolism of both xenobiotics and endogenous substrates, which include androgens, in other species (Gibson et al., 2002). Alligators inhabiting contaminated lakes in Florida exhibit lower plasma T concentrations than alligators collected from a reference site (Guillette et al., 1994, 1999a,b,c; Gunderson et al., 2004). Therefore, it is conceivable that the modulation of hepatic enzymes by xenobiotics present in the serum and tissues of alligators could lead to increased clearance of steroids from the plasma, as has been demonstrated in other species (Bammel et al., 1992; Chen et al., 1993, 1994; Wilson and LeBlanc, 1998). The goals of this study were to first of all determine whether the known and suspected CYP3A inducers dexamethasone, metyrapone, rifampicin, and toxaphene up-regulate the alligator CYP3A77 gene identified in this study as well as six nuclear receptors that are known or suspected to be involved in the transcriptional regulation of hepatic enzymes in other taxa. Secondly we were interested in examining if changes in CYP3A77 gene expression lead to alterations in plasma T concentrations over a relatively short time period. Lastly, we measured basal levels of CYP3A77 and SXR transcripts in wild juvenile alligator populations in Florida to determine whether variation exists among reference populations and populations exposed to organochlorine contamination.

#### 4.1. Up-regulation of alligator CYP3A77 and nuclear receptor genes

Dexamethasone and toxaphene both up-regulated alligator CYP3A77 transcript in yearling female alligators. Metyrapone and rifampicin did not have a significant effect. We had expected metyrapone to act as a strong inducer, as it does in chickens (CYP3A37) (Ourlin et al., 2000). Multiple CYP3A isoforms

Table 4

Simple linear regression analyses examining the relationship between mean relative CYP3A77 expression and SXR (Lakes grouped = Alligators from Lakes Apopka, Orange, and Woodruff grouped together)

Site	$N$	$R^2$	$p$ -value	Equation
Lakes grouped (males and females)	30	0.15	0.03	$\text{Log}(\text{CYP3A77}) = 0.49(\text{log SXR}) + 0.072$
Lakes grouped (females only)	15	0.3	0.03	$\text{Log}(\text{CYP3A77}) = 0.87(\text{log SXR}) + 0.1$
Orange Lake (males and females)	10	0.47	0.03	$\text{Log}(\text{CYP3A77}) = 0.72(\text{log SXR}) + 0.06$
Orange Lake (females)	5	0.76	0.05	$\text{Log}(\text{CYP3A77}) = 1.22(\text{log SXR}) - 0.19$

Only significant relationships are reported ( $p \leq 0.05$ ).

exist within other species and it is possible that metyrapone sensitive CYP3A isoforms exist in alligators. Another possibility to explain these results is that 24 h is either too long or too short a time period to detect the up-regulation of CYP3A77 by metyrapone or rifampicin. Ourlin et al. (2000) reported pronounced differences among inducers in the time required to induce CYP3A37 (chicken). In chickens, metyrapone achieved maximal up-regulation of CYP3A37 in 24 h, whereas phenobarbital required 48 h. More work is needed in alligators to further characterize the regulation of this gene and to conduct a time course for each inducer. This was an initial study to explore the regulation of the alligator CYP3A77 gene and we had a limited number of animals to work with, since the American alligator is a protected species, making a time course for each treatment unfeasible at the time the work was conducted.

The nuclear receptors SXR, glucocorticoid receptor (GR), RXR (retinoid X receptor), and possibly ER, androgen receptor (AR), and PR (progesterone receptor) appear to work together in the receptor dependent regulation of CYP3A in different taxa, and estrogens, androgens, corticosteroids, and progestins are all known to act as substrates and/or inducers of CYP3A and activators of SXR in different model systems (Huss et al., 1996; Wang and Strobel, 1997; Blumberg et al., 1998; Ertl et al., 1998; Reilly et al., 1999; Ertl et al., 1999b; Ourlin et al., 2000; Gibson et al., 2001; Cai et al., 2003; Anakk et al., 2003a). SXR and CYP3A share a broad range of inducers and we were interested in comparing the up-regulation patterns of various nuclear receptors with CYP3A77 in alligators. Potential response elements for estrogen receptors, PR, GR, and SXR have been identified on the proximal promoters of CYP3A isoforms in other taxa suggesting that these nuclear receptors are functionally related to CYP3A gene expression (Jounaidi et al., 1994; Huss et al., 1996; Blumberg et al., 1998; Gibson et al., 2001; Anakk et al., 2003a). In this study, ER $\beta$  was up-regulated after 24 h in alligators exposed to rifampicin and metyrapone but not dexamethasone or toxaphene. None of the other receptors examined in this study exhibited significant changes after 24 h in response to these exposures. It is interesting to note that alligators from two of the six treatment groups exhibited significant positive relationships ( $p \leq 0.05$ ) between CYP3A77 and one of the estrogen receptors although animals from the two treatment groups that demonstrated up-regulated CYP3A77 transcript (dexamethasone and toxaphene) did not exhibit significant relationships between CYP3A77 and ERs. These relationships do not provide a definitive link between CYP3A77 and ERs (as well as the other NRs examined) although regulation of CYP3A by ERs is suggested in other taxa with potential estrogen receptor (ER) response elements being present on the CYP3A promoter region of mice (CYP3A13), rats (CYP3A23), and humans (CYP3A 4, 5, and 7) (Jounaidi et al., 1994; Huss et al., 1996; Gibson et al., 2001; Anakk et al., 2003a) and ER may regulate the female specific patterns of CYP3A9 expression in rats, which is regulated in part by estrogen (Wang and Strobel, 1997; Anakk et al., 2003b). It is also interesting to note that PR exhibited a negative relationship with CYP3A in animals from the toxaphene treatment group. Progesterone decreases the E<sub>2</sub>-induced increases in CYP3A9 and it has been suggested that high plasma progesterone concentrations reached during early pregnancy suppress CYP3A9 message in rats (Anakk et al., 2003b). Furthermore, PR response elements are present on the proximal promoter of human CYP3A isoforms providing additional support for a regulatory role of P<sub>4</sub> and PR in CYP3A transcription (Gibson et al., 2002). In alligators the organochlorine compound, endosulfan, binds the alligator PR receptor (Vonier et al., 1996).

#### 4.2. CYP3A77 and plasma T concentrations

The results from the up-regulation study indicate that toxaphene elevates CYP3A gene expression in alligators and could potentially lead to increased clearance of T. This mechanism for alterations in circulating hormone concentrations has been demonstrated in chickens injected with DDT, which leads to increased cytochrome P-450 content and decreased plasma E<sub>2</sub> concentrations (Chen et al., 1994). However, we observed no changes in plasma T after 24 h in any of the treatment groups. We measured CYP3A77 mRNA and not protein activity and it has been shown that 48–72 h are required for maximal induction of hepatic enzyme activities (CYP1 and CYP2 families) in alligators (Ertl et al., 1998). As discussed earlier we were restricted in the number of animals available for experimentation and therefore examined only the short-term effects of CYP3A77 induction on plasma T concentrations in this study. Future studies are planned to examine later time points.

Examining changes in plasma T over time is complicated by the fact that the hypothalamic-pituitary-gonad axis is presumably intact in these animals. If the axis is still functioning properly, it should help to counteract any increases in plasma T clearance by increasing testosterone synthesis or the protection of circulating T by sex steroid binding proteins and thus maintain hormone homeostasis. Compensatory mechanisms have been demonstrated in humans and rodents injected with hepatic enzyme inducers with increases in urinary clearance of T metabolites but no change in plasma sex steroid concentrations being observed (Bammel et al., 1992; Wilson and LeBlanc, 1998).

Increased plasma steroid concentrations have been reported in relation to exposure to certain CYP3A inducers, which include diazepam (Arguelles and Rosner, 1975), protease inhibitors (Collazos et al., 2002), and rifampicin (Lonning et al., 1989; Bammel et al., 1992). Rifampicin is thought to increase sex hormone binding globulin (SHBG) that serves to protect E<sub>2</sub> from biotransformation and elimination (Lonning et al., 1989). This stresses the importance of measuring multiple endpoints when examining homeostatic mechanisms regulating plasma steroid concentrations. Interestingly, in this experiment rifampicin exhibits a trend towards increasing plasma T after 24 h of exposure, although this increase is not statistically significant ( $p=0.2$ ) due to high variation and possibly the low sample size.

We did not directly test whether the alligator CYP3A77 gene and its enzyme product play a role in the biotransformation of T, although the regression analyses examining the relationship between mean relative CYP3A77 gene expression and plasma T suggest that there is a relationship. Dexamethasone treated animals exhibited a strong positive relationship between mean

relative CYP3A77 gene expression and 24 h plasma T concentrations. SXR exhibited a strong negative relationship with plasma T in animals injected with dexamethasone. No relationships were observed in the toxaphene treatment group (or other groups), which is interesting since dexamethasone and toxaphene both up-regulated CYP3A77 transcript. These results warrant further investigation since we had expected to observe a negative relationship between plasma T and CYP3A77, which would suggest that the up-regulation of CYP3A77 leads to a decrease in plasma T.

#### 4.3. CYP3A77 expression in wild populations

Based on the results from the acute up-regulation portion of this study we examined steady state CYP3A77 and SXR gene expression in juvenile alligators collected from Lakes Woodruff, Orange, and Apopka. Contrary to what we predicted, no differences in CYP3A77 or SXR gene expression were observed among juvenile alligators collected from Lakes Apopka, Orange, or Woodruff. Furthermore, no sexually dimorphic patterns were observed in CYP3A77 gene expression in alligators collected from Lake Woodruff. Sexually dimorphic patterns of total hepatic testosterone hydroxylase activity have been reported in juvenile alligators collected from Lake Woodruff (Gunderson et al., 2001). It is possible that inducers are not present in high enough concentrations in Lake Apopka to elicit an effect or that the expression of CYP3A77 is regulated post-transcriptionally. We injected juvenile alligators to attain a circulating concentration of approximately 21.5 ppm in the plasma and observed up-regulation of the purported CYP3A gene at this concentration in female alligators. The mean toxaphene concentration measured in Lake Apopka eggs collected in 2000 was approximately 9.4 ppm, with concentrations ranging from 0.29 to 40.5 ppm (Woodward et al., 2003). The concentration of toxaphene injected into female yearling alligators in this study falls within the concentration ranges reported in alligator eggs from Lake Apopka, although we do not know how this relates to concentrations stored in the body fat of adult and juvenile alligators or to the ratio of toxaphene circulating in plasma relative to toxaphene stored in body fat.

A lack of variation among lakes in constitutive CYP3A77 expression in wild animals could also be related to stress induced changes in its expression. In this study, we demonstrated that dexamethasone (a glucocorticoid) induces CYP3A77 mRNA. Wild animals in this study were held in cloth bags for 2–7 h before necropsy. Our laboratory group has demonstrated that  $\beta$ -corticosterone concentrations rise significantly within 2 h of capture, which in turn could lead to increased CYP3A77 gene transcription (Guillette et al., 1997; Gunderson et al., 2003). This would mask differences among lakes that might in fact exist. Future studies need to be conducted to examine the effects of capture stress on gene expression. This emphasizes a potential obstacle associated with working with wild alligators since reported observations have a stress response component unless the tissues are collected immediately upon capture. The animals used in the up-regulation portion of this study were captive raised and accustomed to being handled by humans, which min-

imizes the stress-related changes associated with handling the animals.

Temperature and/or season are known to influence the inducibility and activity of cytochromes P-450 enzymes in fishes and turtles, although limited data are available for seasonal changes in cytochromes P-450 enzymes in immature animals (Arukwe and Goksoyr, 1997; Lange et al., 1998, 1999; Rie et al., 2000). Variation in temperature or season could provide an explanation for the lack of differences or up-regulation among sites, although animals were collected from the field in late April, and we observed significant CYP3A77 up-regulation in yearling female yearlings in early June.

#### 4.4. Summary

In this study, we were interested in examining whether known and suspected CYP3A inducers are capable of modulating the alligator CYP3A77 gene as well as nuclear receptors that are implicated in its regulation in other taxa. The regulation of CYP3A77 by organochlorine compounds is of interest because of the role it plays in the biotransformation of both endogenous substrates, such as testosterone, and xenobiotics. The induction of CYP3A77 activity by organochlorine compounds could potentially lead to changes in plasma T concentrations as well as lead to activational alterations in sexually dimorphic patterns of hepatic enzyme expression. We demonstrate that toxaphene, an organochlorine compound present at elevated concentrations in Lake Apopka, up-regulates CYP3A77 in yearling female alligators and thus could activationally modulate the expression of this enzyme in alligators inhabiting contaminated sites. Furthermore, no changes in plasma T were observed 24 h after exposure to CYP3A inducers.

Nuclear receptors transcriptionally regulate the expression of certain hepatic P-450 enzymes as well as a diverse range of hormonally regulated endpoints and we were also interested in examining whether acute exposure to these inducers modulates the expression of ERs, AR, GR, PR, and SXR. ER $\beta$  was up-regulated by rifampicin and metyrapone but not by toxaphene or dexamethasone. None of the other nuclear receptors measured in this study exhibited changes in response to these compounds. Interestingly, the estrogen receptors exhibited the most consistent relationship with CYP3A77 gene expression. These data suggest that estrogen receptors might be related to CYP3A77 expression, which is supported by the fact that potential estrogen receptor binding sites have been identified on the proximal promoter of CYP3A isoforms in rodents and humans (Huss et al., 1996; Anakk et al., 2003a). It is also important to note that PR demonstrated a negative relationship with CYP3A77 gene expression in response to toxaphene exposure, suggesting that these genes could in some way be related in alligators.

Finally, we examined basal CYP3A77 expression in wild populations of juvenile alligators collected from Lakes Apopka, Woodruff, and Orange. CYP3A77 expression was not sexually dimorphic within any of the sites nor did it differ among sites. We had predicted that increased CYP3A77 expression would be observed in Lake Apopka alligators and that sexually dimorphic patterns would be observed in Lake Woodruff animals

(Gunderson et al., 2001). Further studies are needed to examine the relationship between stress, hepatic enzyme expression, and plasma T concentrations in alligators.

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