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Novel cDNA sequences of aryl hydrocarbon receptors and gene expression in turtles (*Chrysemys picta* and *Pseudemys scripta*) exposed to different environments

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Abstract

Reproductive changes have been observed in painted turtles from a site with known contamination located on Cape Cod, MA, USA. We hypothesize that these changes are caused by exposure to endocrine-disrupting compounds and that genes involved in reproduction are affected. The aryl hydrocarbon receptor (AHR) is an orphan receptor that is activated by environmental contaminants. AHR mRNA was measured in turtles exposed to soil collected from a contaminated site. Adult turtles were trapped from the study site (Moody Pond, MP) or a reference site and exposed to laboratory environments containing soil from either site. The red-eared slider was used to assess neonatal exposure to soil and water from the sites. The environmental exposures occurred over a 13-month period. Juveniles showed an age-dependent increase in brain *AHR1*. Juvenile turtles exposed to the MP environment had elevated gonadal *AHR1*. Adult turtles exposed to the MP environment showed significantly decreased brain *AHR2*. The painted turtle AHR is the first complete reptile AHR cDNA sequence. Phylogenetic analysis of the painted turtle AHR showed that it clusters with other AHR2s. Partial AHR1 and partial AHR2 cDNA sequences were cloned from the red-eared slider. MEME analysis identified 18 motifs in the turtle AHRs, showing high conservation between motifs that overlapped functional regions in both AHR isoforms.

Keywords

aryl hydrocarbon receptor; turtles; reptiles; AHR1; AHR2

1. Introduction

1.1 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that contains the characteristic motifs basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) (Kawajiri et al. 2007). AHR was discovered and subsequently cloned using photoaffinity-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Burbach et al. 1992). TCDD is an environmental contaminant that is known as the classical ligand of AHR. TCDD is a potent inducer of cytochrome P4501A1 (CYP1A1), which is also induced by a wide range of

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drugs, dietary agents, and environmental contaminants (Tompkins et al. 2007). AHR binds other synthetic compounds, mainly planar hydrophobic halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (Denison et al. 2003). It has been suggested that that vertebrates evolved dioxin sensitivity as an adaptive response to environmental stressors (Hahn 2002). Evidence suggests that AHR arose in early bilateral metazoans and is present in invertebrate phyla. In vertebrate lineages, it has undergone duplication and diversification, resulting in a family of *AHR* genes (*AHR1*, *AHR2*, *AHRR*). It is possible that the varying degrees of sensitivity to dioxin found among animal taxa stems from the diversification of AHR and differential expression of the AHR types.

When bound to TCDD, AHR dimerizes with AHR nuclear translocator (ARNT), enters the nucleus, and activates transcription via binding to xenobiotic response elements (XRE). When CYP1A1 is activated, AHR/ARNT enters the nucleus and binds XRE in the proximal and distal promoter regions of the CYP1A1 gene (Kawajiri et al. 2007). The binding of the AHR/ARNT heterodimer to the proximal XRE of CYP1A1 results in chromatin remodeling of the CYP1A1 gene and thus increased DNase sensitivity, leading to TCDD-activated gene transcription (Wang et al. 2002). AHR is degraded rapidly *in vivo* and *in vitro* after ligand binding and is ligand-dependent (Roberts et al. 1999). *AHR*-deficient or knockout mice do not show a typical CYP1A1 induction in response to challenge with classical AHR agonists, indicating that CYP1A1 induction is AHR-dependent. Evidence suggests that AHR plays a key role reproductive development by engaging in cross-talk with genes key to reproduction. Both human and mouse CYP19 P450 aromatase genes possess an XRE that binds AHR and ARNT (Baba et al. 2005). The AHR pathway is also known to interact with the estrogen signaling pathway, inhibiting ER signaling through several mechanisms (Safe et al. 2003). In *AHRKO* mice, mRNAs of follicle stimulating hormone and lutenizing hormone receptor are reduced, resulting in reduced ovarian response to gonadotropin injection (Barnett et al. 2007). AHR response elements have been found in the promoter of the proapoptotic gene marker *Bax*, and *Bax* mRNA was shown to increase in the ovaries of mice after treatment with the AHR ligand dimethylbenz(a)anthracene (Matikainen et al. 2001).

To date, there are few studies on reptile AHR. Hahn et al. (1994) established that AHR was present in the painted turtle (*Chrysemys picta*), via photoaffinity labeling using hepatic cytosolic fraction and [¹²⁵I]-N₃Br₂DD. A specifically labeled protein was not observed in the American alligator (*Alligator mississippiensis*), although only one individual was examined (Hahn et al. 1994). A complete reptile AHR has not been sequenced before, although partial AHRs have been determined for a phylogenetic study on several species of turtle (Barley et al. 2010).

1.2 Massachusetts Military Reservation (MMR)

The MMR is a military training site on Cape Cod that was declared an Environmental Protection Agency Superfund site in 1989. The site has several sources of contamination that have contaminated the groundwater in the area (AFCEE 2003). The study site Moody Pond is located close to the MMR, while the reference site Washburn Pond is located away from the path of groundwater flow.

Moody Pond sediment elutriate has been shown to have similar effects to TCDD in zebrafish embryos. Expression of a novel aromatase B splice variant was shown to increase with exposure to TCDD (Novillo-Villajos 2003). A similar expression pattern was induced in embryos exposed to Moody Pond sediment elutriate, but not when embryos were exposed to Moody Pond water. In female painted turtles from Moody Pond, reproductive changes such as reduced ovarian follicular reserve, reduced plasma estradiol and vitellogenin have been described. Exposure to environmental contaminants is the likely cause of these changes (Rie et al. 2005). In male painted turtles from the Moody Pond study site, Kitana et al.

(2007) observed a decrease in testicular weight, seminiferous tubule diameter and epididymal sperm number. A previous study examining painted turtles trapped from Moody Pond found hepatic biotransformation enzymes to be elevated, notably CYP1A1 protein, suggesting contamination of Moody Pond with organic compounds such as dioxin-like compounds (Rie 2000). These studies suggest that both male and female turtles are sensitive to EDC present at this site.

In this study, we cloned the turtle AHR in order to pursue a possible role of AHR/estrogenic pathway interactions associated with the previously observed reproductive deficits. Quantitative changes in AHR mRNA were measured under different environmental conditions.

2. Materials and Methods

2.1 Adult painted turtles

The specific chemical environment of the contaminated site, Moody Pond, has not been defined, but it is located 500 meters downgrade from at least 8 contaminated sites within the MMR. These include fuel and chemical spills, storm drains, and landfills (AFCEE 2003). The pond is also immediately to the east of a well-documented contaminant plume from the MMR (Eastern Briarwood). The reference site, Washburn Pond, is to the east of the MMR and is unaffected by groundwater flows from the MMR. Painted turtles (*Chrysemys picta*) were collected from sites in Mashpee, MA for a 13-month laboratory exposure to sediment from the contaminated site or the reference site (Moody or Washburn Pond, respectively) during two one-week periods in both June and October 2005 using hoop traps baited with canned sardines or cat food. The crossover experiment (Fig. 1a) was done with the painted turtle adults to determine whether 1) the effects of a Moody Pond origin could be mitigated by exposure to the reference environment, Washburn Pond, or 2) if an individual from a Washburn Pond origin would be impacted by a yearlong exposure to the Moody Pond environment. A subset of individual males and females trapped at Moody Pond were placed in the Washburn environment tanks; also a subset of Washburn Pond individuals were placed in the Moody environment tanks. Turtles trapped in Washburn and Moody Ponds were also placed into their native environments. At the beginning of the experiment, 9 adult male and 6 adult female painted turtles of Washburn origin were exposed to the Moody environment; 2 adult male and 4 adult females of Moody origin were exposed to the Washburn environment. Eight adult male and 8 adult female painted turtles of Washburn origin were exposed to the Moody environment; 1 adult male and 3 adult females of Moody origin were exposed to the Moody environment.

2.2 Juvenile red-eared sliders

Due to a lack of commercially available painted turtle neonates, red-eared slider (*Pseudemys scripta*) neonates were purchased from Robert Clark (Hammond, Louisiana, USA). A species of pond turtle that is closely related to the painted turtle, red-eared sliders were used to determine effects of contaminated soil on turtles exposed early in life, starting as neonates (Fig. 1b). To distribute sexes equally between tanks, regression analysis of the ratio between precloacal length and the two posterior lobes of the plastron (PPR) was done. PPR can be used to approximate sex in neonate turtles (Lovich et al. 1992; de Solla et al. 1998). Males have a relatively longer precloacal length (Lovich et al. 1992). A group of neonates was autopsied for sample collection at the start of the experiment for assessment of sex dimorphic characteristics and gene expression. At the beginning of the experiment, 30 neonate red-eared sliders were placed in the Washburn environment tank, with the regression analysis assessing 15 female and 15 male red-eared sliders. Twenty-nine red-

eared slider turtles were placed in the Moody environment tank, of which 14 were female and 15 were males.

2.3 Animal maintenance and experimental design

Turtles were maintained in the Laboratory Animal Care Facility of the Department of Biology at Boston University. The experimental animals were placed in tanks starting in November 2005 and sacrificed in December 2006 for a 13-month period. Soil for the exposure was collected from Moody and Washburn Pond and stored for later use at 4 °C in doubled plastic bags closed with zip ties.

Painted turtle adults were placed in large floor-standing fiberglass or stainless steel tanks, while neonate red-eared sliders were maintained in Lucite aquaria. The tanks were divided, with standing water in one section and sediment in the other. The water in the tank was pumped onto the surface of the soil to ensure a constant flow of water through the sediment. Water was changed every week in the adult tanks, and every two weeks in the neonate tanks. Animals were given a 12-hour day cycle and fed *ad libitum* daily. The soil in both adult and neonate tanks was replaced every two months. Due to the logistical problem of transporting and storing large quantities of collected water from the study site, adults were exposed only to the sediment collected from each site. Neonates were exposed to both soil and water collected from the field sites. Adult tanks were supplied with tap water filtered for chloramines. The water collected from the ponds was filtered prior to storage and stored at 4 °C in doubled plastic bags closed with zip ties.

At sacrifice in December 2006 after 13 months of exposure, all animals were anesthetized in ice slurry and decapitated. Tissue samples were flash-frozen. In neonate turtles, sex can be visually assessed by examining the gonad (Crews et al. 1991). Weight and morphometric measurements were taken at autopsy. Ovarian follicle diameter was measured in adults.

Remaining adults at the end of the environmental exposure experiment were counted as follows: 8 Washburn origin males, 1 Moody origin male, 4 Washburn origin females and 4 Moody origin females in the Washburn environment. From the Moody environmental exposure, there were 8 Washburn Pond origin males, 4 Washburn origin females and 3 Moody origin females. Thus during the 13 months from the Washburn tank: 1 Washburn male and 2 Washburn females were lost; 1 Moody origin male was lost. From the Moody tank, 4 Washburn origin females were lost and 1 male Moody origin was lost; no Moody origin females were lost. There was not a sufficient number of Moody origin males obtained for the minimum sample size and statistical analysis.

At the termination of the juvenile experiment, a total of 17 Washburn females, 6 Washburn males, 17 Moody females and 3 Moody males were sacrificed. Mortality after 12 months of laboratory exposure for the Washburn tank was 7 turtles and for the Moody tank 9 turtles. Of the neonate group, 5 males and 8 females were sacrificed at time of receipt of the animals from the supplier. Analysis of survival times did not reveal significant differences between the groups.

2.4.1 Molecular cloning of AHRs—Total RNA was extracted with TRI reagent (Sigma). PCR products were resolved on 1% agarose gels and visualized over a UV light source. Invitrogen Superscript III was used in reverse-transcriptase reactions with oligo dT primer. The following PCR conditions were used: 94 °C opening for 5 min followed by 5 cycles of 94 °C for 30 s, 43 °C for 45 s, 72 °C for 60 s, followed by 40 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. Sequence was confirmed by direct sequencing of end-to-end clones.

2.4.2 Painted turtle AHR2—Degenerate primers D33F and D348R (courtesy of Dr. Mark Hahn, Woods Hole Oceanographic Institution) were used to amplify a 967 bp fragment from painted turtle ovarian tissue (Table 1). PCR conditions were as follows: 94 °C opening for 5 min followed by 5 cycles of 94 °C for 30 s, 65 °C for 60 s, followed by 5 cycles of 94 °C for 30 s, 60 °C for 60 s, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 60 °C for 60 s, followed by a final extension at 68 °C for 10 min. The 5' and 3'-ends of the cDNA fragment were extended using RACE (Invitrogen Generacer). 5' RACE cDNAs were made with Invitrogen Thermoscript, a high-temperature reverse transcriptase for difficult cDNA templates. RACE PCRs were done under the following conditions: 94°C opening for 5 min, 5 cycles of 94 °C for 30 s followed by 74 °C for 3 min, 5 cycles of 94 °C 30 s followed by 72 °C for 3 min, then 25 cycles of 94 °C for 30 s, followed by 68 °C for 30 s, followed by 72 °C for 3 min, with a final extension at 72 °C for 10 min. Because the painted turtle *AHR* cDNA had high sequence identity with the cormorant AHR, a 5'RACE primer (AHR BFA5'4) that was originally designed from cormorant AHR was used (Yasui et al. 2004).

Two contiguous AHR sequences that did not overlap were obtained. Four primers were designed (AHR5-AHR8) and used to clone two overlapping segments of *AHR*. The two fragments were digested at a unique restriction site located in the overlap using *BsaI* (New England Biolabs). After the digest, the two halves were ligated and the clones sequenced (primers seq1-seq14, Table 1) to obtain a contiguous sequence. Attempts to design primers that would obtain an end-to-end clone were not successful.

2.4.3 Cloning of the red-eared slider AHR cDNAs—The degenerate AHR primers D33F and D348R were used to amplify transcripts from red-eared slider hepatic tissue (Table 1). The PCR product was amplified as described above. The initial amplification yielded an approximately 1,000 bp fragment.

Sequencing primers (seq1- seq4, Table 2) were designed to sequence the fragment, yielding two contiguous AHR sequences. These sequences had similarity with AHR1 or AHR2 after a BLAST search. These AHR sequences were then extended using 5'RACE and 3'RACE.

2.5 Primer validation for quantitative PCR

Primers were designed using PrimerExpress (ABI) to span exon boundaries estimated from homologous human gene sequences. Specificity of the qPCR primers was verified by sequence analysis. Tissue samples were pooled only for the gonadal tissue of female red-eared sliders, while male gonads were not pooled because of the limited sample size. Neonate gonads were not analyzed due to insufficient size of the tissue samples obtained. Amplification efficiency values and primer concentrations were optimized prior to assay.

The painted turtle *AHR2* qPCR primers q181F and q314R are located in the conserved basic 2 region of *AHR* (Table 1). Primers for actin, the reference gene used to normalize painted turtle *AHR*, were obtained by cloning a short sequence withobtained from a previously published paper (Valenzuela et al. 2007). Actin primers (actin F and actin R) were designed and validated as described above for each tissue measured in the experiment (Table 1).

Attempts to validate qPCR primers from the red-eared slider *AHR2* sequence were not successful. qPCR primers were successfully validated from the red-eared slider *AHR1* sequence (Table 2). The red-eared slider *AHR1* primers, qPSAHR1-66F and qPSAHR1-150R are located in the basic 1 region of *AHR*, as estimated by alignments with other known AHR sequences. Primers PP1F and PP1R, for the reference gene protein phosphatase 1 γ , were previously published by Ramsey et al (2007).

Tissue samples were pooled only for the gonadal tissue of female red-eared sliders. Male red-eared slider gonads were not pooled because of the limited number of males available for analysis. Neonate gonads were not analyzed due to insufficient sample size. SYBR Green Master Mix (ABI) was used in a 30 μ L reaction pipetted in triplicate in ABI 384-well qPCR plates. Real-time qPCR experiments were conducted on an ABI Prism 7900 real-time PCR machine with the following parameters: 50 °C for 2 min followed by 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s.

A 4 pmol concentration of painted turtle AHR primers and 3 pmol concentration for the painted turtle actin primers were used. AHR2 primers were used at a 6 pmol concentration for painted turtle testis qPCR measurements. A 5 pmol primer concentration was used for red-eared slider AHR1 and 3 pmol concentration used for red-eared slider PP1.

3.1 Data analysis

qGene was used to normalize the data obtained (Simon 2003). Relative quantification was performed by a modified comparative critical threshold (CT) method that corrects for different PCR amplification efficiencies among primer pairs (Simon 2003). Normalized gene expression is given as mean normalized expression (MNE) = $(E_{PP1}^{\text{meanCTPP1}})/E_{ER\alpha}^{\text{meanCTER}\alpha}$ where E = PCR efficiency ($E = 10^{-(1/\text{slope})}$) and meanCT is the average CT from the three replicates (Pfaffl 2001). For painted turtles and red-eared sliders, actin and PP1 were used to normalize qPCR results, respectively. Data points were rejected if the %SEM was greater than 20%. A minimum of 5 data points were obtained from at least 2 cDNA libraries. The average MNE was determined for each set of replicates obtained from an individual animal, and standard deviation calculated for each MNE. The MNEs were then averaged for each group analyzed, and these values were used to calculate fold change. Fold change was calculated by dividing the average MNE of a given treatment group by the average MNE of the control group.

Statistical analysis (one-way and two-way analysis of variance, ANOVA) was done using SigmaStat 3.5 (SYSTAT). Statistical probability of $P < 0.05$ was considered significant.

3.2 Phylogenetic analysis and sequence alignment

We used maximum likelihood analysis to assess the evolutionary relationship of painted turtle and red-eared slider AHR2s and AHR1 with a combination of other known AHR sequences. Sequences were gathered from NCBI (<http://www.ncbi.nlm.nih.gov/>) and aligned using ClustalW under default settings (Larkin et al. 2007). The alignment was trimmed to remove all gaps and submitted to RAxML BlackBox, where maximum likelihood analysis was run for 100 boot straps (Stamatakis et al. 2008). The trees were then visualized and illustrated using FigTree v1.1.2. (<http://tree.bio.ed.ac.uk/software/figtree/>).

Accession numbers of sequences used in phylogenetic analysis are as follows: zebrafish (*Danio rerio*) AHR1 β and AHR2 (NP_001019987, CAK11168); killifish (*Fundulus heroclitus*) AHR2 (AAC59696); chicken (*Gallus gallus*) AHR1 (AAF70373); human (*Homo sapiens*) AHR1 (AAH69390); gray-tailed opossum (*Monodelphis domestica*) AHR2 (XP_001365696); rainbow trout (*Oncorhynchus mykiss*) AHR2 α (AAC95335); platypus (*Ornithorhynchus anatinus*) AHR2 (XP_001513826.1); cormorant (*Phalacrocorax carbo*) AHR1 and AHR2 (BAD01477, BAF64245.1); black-footed albatross (*Phoebastria nigripes*) AHR1 and AHR2 (BAC87795, BAC87796); Atlantic salmon (*Salmo salar*) AHR2 γ (AAL12247); pufferfish (*Takifugu rubripes*) AHR2 α (AAY96631); African clawed frog (*Xenopus laevis*) AHR1 β (AAV49748).

AHR sequences were aligned using ClustalW 2.0 (Chenna et al. 2003). The partial AHR sequences from red-eared slider and the painted turtle AHR2 were aligned with chicken, zebrafish, and cormorant AHRs. Accession numbers for these sequences are listed above.

3.3 Protein motif identification

AHR amino acid sequences were evaluated using MEME (Multiple Expectation Maximization for Motif Elicitation; <http://meme.nbcr.net>) (Bailey et al. 1994). The AHRs selected for MEME analysis were the same as those used in the phylogenetic analysis discussed above, with 3 additional sequences from invertebrates as follows: scallop (*Chlamys farreri*) AHR (**227908937**); softshell clam (*Mya arenaria*) AHR (**7839662**); clam (*Venerupis philippinarum*) AHR (**295149355**). Motifs were identified using the following settings: maximum number of motifs=20; occurrences of a single motif= any number; minimum length of motifs= 25; maximum length of motif= 300.

4. Results

4.1.1 Painted turtle AHR2 cDNA sequence—The painted turtle *AHR* cDNA sequence (**GQ141218**) is 3,498 bp long with an open reading frame from 176 to 3,283, which contains the AHR motifs bHLH and PAS domains (Fig. 2). Painted turtle *AHR2* had the highest identity (92%) to a predicted protein sequence from platypus (**XP_001513826.1**).

4.1.2 Semiquantitative RT-PCR analysis of painted turtle AHR2—

Semiquantitative RT-PCR analysis of painted turtle AHR2 tissue distribution was done using primers AHR7/AHR8, which amplified a partial sequence of the painted turtle AHR2 from position 1,458 to 2,729. A partial AHR sequence was amplified because no full-length clone primers have been successfully designed. A second, smaller fragment was amplified from female muscle tissue cDNA (Fig. 3). This may indicate the presence of an alternatively spliced isoform found in muscle.

4.2 Red-eared slider AHR sequences

The red-eared slider partial *AHR1* cDNA sequence is 1,102 nucleotides long and encoding 348 aa (**HM640941.1**); the partial AHR2 sequence (**HM640942**) is 1,218 nt long encoding a 351 aa protein. The red-eared slider AHR1 fragment contains the PAS motif, the AHR2 fragment contains the HLH and PAS motifs. These turtle AHRs represent the first reptile AHR isoforms cloned from the same species. The % identity of the painted turtle AHR2 was 98% with the partial red-eared slider AHR2 and 61% with the red-eared slider AHR1. These two AHR fragments are not complete cDNAs, as neither cDNA includes a stop codon.

4.3.1 Alignment of AHR sequences—The alignment of amino acid sequences showed that the AHR is highly conserved in the 5' region (Fig. 4).

4.3.2 Phylogenetic data—An unrooted neighbor-joining tree shows the turtle AHR2s fall into two sister clades. Turtle AHR2s cluster with marsupial and bird AHR2s, while fish AHR2s form a sister clade (Fig. 5). AHR1s form a clade separate from AHR2s. Bird AHR1s form a sister clade with amphibian, human, and the partial red-eared slider AHR1.

4.4 MEME analysis

We identified 18 motifs present in the turtle AHRs. Motifs were included if they were present in at least two of the AHR sequences analyzed. Fifteen phylogenetically diverse taxa were analyzed, including 3 invertebrates. Six motifs (motifs 1, 3–7) were conserved among the majority of the AHRs, which included invertebrate AHRs, AHR1, and AHR2. The motif

architecture shows strong similarities in the 5' portion of both AHR isoforms, with diverging motifs in the 3' portion of the AHRs. Motifs 1 and 2 overlapped the bHLH domain. Motifs 2 and 3 overlapped the PAS-A domain, while 4 and 5 overlapped the PAS-B domain. Motifs 6 and 7 were found in both isoforms of AHR, although not in the turtle or avian AHR1s shown here (Fig. 6). Motifs 8 through 10 were specific to AHR2s. Motif 10 was found only in the albatross and cormorant AHR2s, and motif 11 was found only in fish AHR2s. Motifs 12 through 18 were found only in AHR1s. Motif 10 was found in only two AHR2s from the cormorant and albatross, and motif 11 was only found in fish AHR2s. Motif 12, which is unique to AHR1, is located in the 5' region of AHR1 and overlaps the bHLH. As only a partial red-eared slider turtle AHR1 was obtained, a full-length cormorant AHR1 is shown here (Fig. 6).

4.5.1 Painted turtle brain AHR2 mRNA—One-way ANOVA determined a significant decrease in brain *AHR2* mRNA in the Moody environmental exposure compared to the Washburn group (Fig. 7a, ANOVA, $H = 5.48$, $df = 1$, $P = 0.019$). When analyzed by sex, females from the Washburn origin, Washburn environment had significantly higher brain *AHR2* mRNA than females that were exposed to Moody Pond by origin or environment (Fig. 7b, ANOVA, $F = 45.9$, $df = 3$, $P < 0.001$).

4.5.2 Other painted turtle tissues—*AHR2* mRNA was measured in painted turtle hepatic, ovarian, and testicular tissues (data not shown). No significant changes were observed.

4.6.1 Red-eared slider brain AHR1 mRNA—Brain *AHR1* mRNA in the red-eared slider was significantly higher in both groups of juveniles when compared to neonates (ANOVA, $F = 9.3$, $df = 2$, $P < 0.001$), however no environmental effect was observed (Figure 8a). When analyzed by sex, the significant difference persisted between female juveniles and neonates (Figure 8b), with juveniles having significantly higher brain *AHR1* mRNA than neonate females (ANOVA, $F = 9.3$, $df = 2$, $P < 0.001$).

4.6.2 Red-eared slider gonadal AHR1 mRNA—Analysis with one-way ANOVA determined significantly higher gonadal *AHR1* mRNA in the Moody environmental exposure group (Fig. 9a, ANOVA, $F = 7.3$, $df = 1$, $P = 0.016$). When analyzed by sex using one-way ANOVA (Fig. 9b), the Moody exposed females had significantly higher gonadal *AHR1* mRNA (ANOVA, $F = 10.8$, $df = 1$, $P = 0.009$), while males showed no significant differences in *AHR1* mRNA. The Moody male gonads show a nonsignificant, similar trend of elevated *AHR1* mRNA.

4.6.3 Red-eared slider hepatic tissue—No significant differences were found in red-eared slider hepatic *AHR1* mRNA (data not shown).

5. Discussion

The painted turtle AHR represents the first full-length reptilian AHR cDNA and falls into the AHR2 clade. Cloning AHR1 from painted turtle with primers from a previous study on avian AHR1 was not successful (Yasui et al. 2004).

Partial AHR1 and AHR2 cDNA sequences were determined in the red-eared slider.

AHR2 was quantified in adult painted turtles and AHR1 in neonate and juvenile red-eared slider turtles. Quantitative PCR primer pairs designed to amplify AHR2 in red-eared slider were tested for primer validation, however none of the primers designed were successful.

Extending the red-eared slider AHR2 sequence would likely yield a better option for qPCR primer design.

In an unrooted phylogenetic tree comparing several vertebrate AHRs (Fig. 5), the partial red-eared slider AHR1 clusters with human and amphibian AHR1s. The turtle AHR2s cluster with avian, marsupial, and monotreme AHR2s and form a sister clade to teleost fish AHR2s. Partial *AHR1* cDNA sequences have been identified in 19 turtle species, including the painted turtle (Barley et al. 2010). There appears to be only one AHR isoform in most mammalian species, however the gray-tailed opossum has AHR1 and AHR2 (Dr. Mark Hahn, personal communication). The platypus has an AHR that is closest to AHR2. The platypus is noted for retaining the avian/reptilian character of oviparity and has a mix of reptilian and mammalian characteristics both physiologically and genetically (Warren et al. 2008).

MEME analysis identified 18 motifs common to the AHRs analyzed (Fig. 6). Motifs 1–5 indicate that the N-terminus is highly conserved between both AHR isoforms. The motifs identified in the N-terminus half of both AHR isoforms overlapped known functional domains. The C-terminal halves of AHR are highly variable except in closely related species. Most of the motifs specific to the different AHR isoforms were located in the Q-rich variable region, on the 3' region of the AHR. Four motifs appear to be unique to AHR2s, and six motifs were unique to AHR1s. The partial red-eared slider turtle AHR1 had four motifs in common with the cormorant AHR1. Certain motifs may be unique to certain taxa and isoforms. Motif 10 was found only in avian AHR2, and motif 11 was only found in fish AHR2s. The red-eared slider AHR1 was only a partial cDNA and did not include motif 12, which overlaps the bHLH domain. The regions of AHR known to be important in AHR function are the bHLH and PAS domains, located in the N-terminal half of AHR (Dolwick et al. 1993). Differences in sequence identity may point to differences in functionality of the expressed AHR protein.

It is not known which AHR isoform is dominant in reptiles. AHR2 has been reported in birds and fish (Yasui et al. 2004). Most mammals have one AHR, which is of the AHR1 lineage (Hansson et al. 2007). AHR is ubiquitously expressed in almost all tissues in humans and in fetal tissue, and is relatively high in both the testis and ovary in adult humans (Yamamoto et al. 2004). Both AHR isoforms are hypothesized to have arisen before the divergence of fish and avian lineages, with AHR2 lost in some mammalian lineages (Hahn et al. 1997; Hahn 2002; Yasui et al. 2004). Fish have more AHR genes than other vertebrates (Hahn et al. 2006). Several studies have determined that AHR2 is the predominant form in fish (Roy et al. 1997; Abnet et al. 1999; Karchner et al. 1999; Tanguay et al. 1999). At least six AHR genes have been found in salmon, two are AHR1 and four are AHR2 (Hansson et al. 2003; Hansson et al. 2004). It has been suggested that the sensitivity of salmonids to TCDD is partially due to the existence of multiple AHRs (Hansson et al. 2007). qPCR measurements showed that the different salmon AHRs were differentially expressed in tissues and had differential sensitivity to TCDD (Hansson et al. 2007). In zebrafish, AHR1A cannot bind TCDD or activate transcription, while AHR1B has specific, high-affinity binding of tritiated TCDD (Andreasen et al. 2002; Karchner et al. 2005).

An *in vitro* reporter gene transactivation assay indicated that both AHR isoforms are transcriptionally active in two avian species (Yasui et al. 2007). *AHR2* was found to have reduced transcriptional efficiency as compared to *AHR1* after transfected cells were exposed to TCDD (Yasui et al. 2007). Yasui et al. (2007) determined cormorant tissue expression profiles using quantitative PCR. Levels of *AHR1* expression were similar in most tissues. Hepatic *AHR1* mRNA was expressed at significantly higher levels than *AHR2* (Yasui et al.

2007). A significant sex difference was observed in cormorant hepatic *AHR2* mRNA but not for *AHR1*, and no age-dependent expression of *AHR* was found in hepatic tissue. These data suggest that the dominant avian form of AHR might be *AHR1*, unlike fish, in which the dominant form appears to be *AHR2*. Semiquantitative PCR analysis of tissue distribution, which utilized primers that amplified a partial sequence of the painted turtle *AHR2* amplified a second, smaller band amplified from female muscle cDNA. The sequencing of the smaller band yielded two contiguous sequences that do not overlap. This transcript of a different size may indicate the presence of an alternatively spliced isoform in muscle. However, more individuals must be examined to determine whether this is truly a sex-specific difference. AHR splice variants have been observed in Wistar rats, which are resistant to dioxin because of their mutated AHR (Moffat et al. 2007).

We hypothesized that the observed changes in *AHR* mRNA associated with exposure of turtles to the Moody Pond environment might be the cause of the observed reproductive effects, possibly by affecting AHR-ER crosstalk. There are several proposed mechanisms of crosstalk between AHR and ER signaling pathways (Safe et al. 2003). The AHR is thought to be constitutively active in many cases, although there is evidence that AHR expression in fish can be affected by exposure to environmental contaminants. Killifish from Newark Bay, NJ, a site contaminated with wastewater effluent, had decreased hepatic vitellogenin mRNA and protein, and high basal levels of *CYP1A* mRNA and protein expression (Bugel et al. 2010). In zebrafish exposed to the AHR agonists benzo-a-pyrene or TCDD in combination with the AHR antagonists resveratrol and α -naphthoflavone, differential tissue responses were observed (Bugiak et al. 2009). TCDD induced hepatic and vascular *CYP1A* mRNA, while benzo-a-pyrene did not elicit a change (Bugiak et al. 2009). Benzo-a-pyrene induced *CYP1A* in mesenteric artery (Bugiak et al. 2009).

In the gonads of juvenile turtles exposed to the Moody Pond environment, *AHR1* mRNA was significantly elevated (Fig. 9). This difference persisted when females alone (but not males) were analyzed. The observed changes in gonadal *AHR1* mRNA in juvenile turtles may be due to exposure to the dioxin-like contaminants that appear to be present in Moody Pond sediment. Brain *AHR1* mRNA expression was significantly higher in juvenile than neonate red-eared sliders, suggesting a possible age-dependent difference (Fig. 8). Exposure to the Moody Pond environment had no effect on *AHR1* mRNA expression in the juvenile turtle brain. Age-dependent changes in AHR expression have not been well studied, although one study has found that AHR in male rat hypothalamus is significantly increased between gestational day 20 and postnatal day 5, as measured by band intensity from semiquantitative PCR (Pravettoni et al. 2005).

In Moody environment-exposed adult turtles, brain *AHR2* mRNA was significantly lower than in Washburn-exposed adults (Fig. 7). No sex dimorphic differences were found. When analyzed by sex, no significant differences were found between males. In the adult females, brain *AHR2* mRNA expression was significantly lower in females that were exposed to the Moody Pond environment and/or the Moody Pond origin. This suggests that the decrease in brain *AHR2* mRNA is a long-term effect, one that persists for at least one year. A similar long-term effect by the Moody environment on hepatic *ER* mRNA was observed in females from the same experiment (data not shown here). The changes in brain *AHR* mRNA could potentially have an effect on sex behavior in the affected turtles. No significant differences were found in hepatic *AHR* mRNA, although a nonsignificant trend towards elevated hepatic *AHR2* in adults was observed (data not shown). No differences were seen in ovarian or testicular expression of *AHR2* mRNA in adult turtles exposed to the Moody Pond environment (data not shown).

The observed changes in *AHR* mRNA do not indicate whether the AHR pathways, such as CYP1A1, are affected, and it is not known whether AHR alone is a useful biomarker of exposure to environmental contaminants. CYP1A1 expression was not examined in this study. Further, the sample size, while sufficient for biological replicates, is limited. However, the observed changes to AHR in animals exposed to the Moody Pond environment merit further investigation.

The cDNA sequences of painted turtle AHR2 and partial red-eared slider AHR1 and AHR2 were determined and several motifs common to AHRs determined using MEME analysis. As these sequences are novel, future functional studies will determine whether the dominant AHR isoform in reptiles is AHR1, similar to avians. Reptiles are an evolutionary intermediate between mammals and fish, and thus the study of reptile AHRs will aid in understanding the evolution of AHRs in vertebrates. We also identified a potential change to *AHR2* mRNA caused by exposure to Moody Pond sediment in adult brain tissue. Exposure to Moody Pond sediment and water in juvenile turtles affected gonadal *AHR1* mRNA. Future studies on these effects in turtles should determine if there is a relationship between AHR mRNA expression and CYP1A1 induction.

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Figure 1.

Diagram of experimental design. (a) Adult painted turtles trapped from Moody Pond or the reference site Washburn Pond were exposed to mimicked environments representing either their native environment or the foreign environment in the laboratory. The purpose of the “crossover” was to determine if gene expression profiles were similar in animals exposed to the same environment in the laboratory regardless of their origin site. Environmental exposure tanks contained soil from the study or reference site. Water from the sites could not be collected due to the logistical problem of storing large volumes of water for twice-weekly tank cleaning. (b) Neonate red-eared slider turtles were used to assess exposure of young animals to the study site or reference environments containing soil and water from the respective sites.

Figure 3.

Semiquantitative PCR tissue distribution of painted turtle *AHR2*. PCR primers were used to amplify a portion of *AHR2* (position 1,458 to 2,279) in cDNA libraries from several painted turtle tissues. Samples from one male and one female from the reference site (Washburn Pond) are shown. The arrow indicates a second, smaller transcript that was amplified in muscle.

Figure 4.

Alignment of turtle AHR amino acid sequences. The amino acid sequences of the AHRs were aligned using ClustalW 2.0 (Chenna et al., 2003). Numbers for painted turtle AHR are shown on the right. Domains were estimated based on alignments of avian AHRs (Yasui et al., 2007). Functional domains are labeled and the ligand-binding domain marked with an asterisk at each end. The complete painted turtle AHR2 is shown with the partial sequences for red-eared slider AHR1 and AHR2. Amino acids identical to the painted turtle AHR2 are boldfaced. Accession numbers are as follows: painted turtle AHR2 (**GQ141218**); red-eared slider AHR1 (**HM640941.1**) and AHR2 (**HM640942**); cormorant AHR1 (**BAD01477**) and AHR2 (**BAF64245.1**).

Figure 5.

Phylogenetic analysis of AHR amino acid sequences. Sequences were gathered from NCBI (<http://www.ncbi.nlm.nih.gov/>) and aligned using ClustalW under default settings (Larkin et al., 2007). The alignment was trimmed to remove all gaps. It was then submitted to RAxML BlackBox, where maximum likelihood analysis was run for 100 bootstraps (Stamatakis et al., 2008). Accession numbers are as follows: zebrafish (*Danio rerio*) AHR1 β (**NP_001019987**) and AHR2 (**CAK11168**); killifish (*Fundulus heroclitus*) AHR2 (**AAC59696**); chicken (*Gallus gallus*) AHR1 (**AAF70373**); human (*Homo sapiens*) AHR1 (**AAH69390**); gray-tailed opossum (*Monodelphis domestica*) AHR2 (**XP_001365696**); rainbow trout (*Oncorhynchus mykiss*) AHR2 α (**AAC95335**); platypus (*Ornithorhynchus anatinus*) AHR2 (**XP_001513826.1**); cormorant (*Phalacrocorax carbo*) AHR1 and AHR2 (**BAD01477**, **BAF64245.1**); black-footed albatross (*Phoebastria nigripes*) AHR1 and AHR2 (**BAC87795**, **BAC87796**); Atlantic salmon (*Salmo salar*) AHR2 γ (**AAL12247**); pufferfish (*Takifugu rubripes*) AHR2 α (**AAV96631**); African clawed frog (*Xenopus laevis*) AHR1 (**AAV49748**). The dotted line surrounds all of the AHR2s analyzed. The scale bar indicates 0.01 expected amino acid substitutions per site.

Figure 6.

Motif architecture of AHR amino acid sequences using Multiple Em for Motif Elicitation (MEME) analysis. Motifs (boxes) and inter-motif regions (horizontal lines) are drawn to scale. Conservation between motifs is indicated by vertical lines connecting the various motifs in common. Horizontal lines indicate the correspondence between the motifs and the basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) functional domains. Certain motifs were found to be specific to taxa, for example, motif 10 was only identified in avian AHRs. As a full-length reptile AHR1 has not yet been sequenced, a bird AHR1 (from the cormorant) is shown for comparison with the partial red-eared slider AHR1. Eighteen motifs were identified among all the AHRs compared and are listed in Table 3. The white motifs are unique to AHR1s and the black motifs are unique to AHR2s. Motifs 6 and 7 do not occur in the AHR1s shown in Figure 7, but were found in the other AHR1s analyzed. The sequences used in phylogenetic analysis in addition to 3 basal AHRs from invertebrates were used in the MEME analysis. Sequence accession numbers are listed in Materials and Methods.

Figure 7.

Graph of mean normalized expression obtained from qPCR measurements of painted turtle brain *AHR2* mRNA according to environmental exposure. “Environment” represents the environmental exposure, either to Moody (M) Pond sediment or Washburn (W) Pond sediment. “Origin” represents the pond where turtles were originally trapped, thus W/M indicates a Washburn origin turtle exposed to the Moody environment. The upper panel shows all individuals from the experiment, the lower panel shows individuals separated by sex. Data are represented as mean $\pm$ standard error of the mean. “A” indicates a statistically significant difference from “B” (ANOVA, $P < 0.05$).

Figure 8.

Graph of mean normalized expression obtained from qPCR measurements of red-eared slider brain *AHR1* mRNA according to environmental exposure. “Environment” represents the environmental exposure, i.e., exposure to either Moody Pond sediment and water, or Washburn Pond sediment and water. The upper panel shows all individuals from the experiment, the lower panel shows individuals separated by sex. Neonates were turtles sacrificed prior to starting the experiment. Data are represented as mean $\pm$ standard error of the mean. “A” indicates statistically significant difference from “B” (ANOVA, $P < 0.05$).

Figure 9.

Graph of mean normalized expression obtained from qPCR measurements of red-eared slider gonadal *AHR1* mRNA according to environmental exposure. “Environment” represents the environmental exposure, i.e., exposure to either Moody Pond sediment and water, or Washburn Pond sediment and water. The upper panel shows all individuals from the experiment, the lower panel shows individuals separated by sex. Data are represented as mean $\pm$ standard error of the mean. “A” indicates statistically significant difference from “B” (ANOVA, $P < 0.05$).

Table 1

Primers used in cloning the painted turtle AHR cDNA sequences. Primers are listed in the chronological order of use in cloning. Forward primer (F), reverse primer (R), sequencing primer (seq), rapid amplification of cDNA ends (RACE), nested primer (nest), quantitative PCR primers (q).

Oligo/Direction		Sequence (5' to 3')	Position	Reference
D33F	F	GTCTAACCCITCIAAGMGIC	354–374	courtesy Dr. Mark Hahn
D348R	R	CATICCRCTYTCICCI GTYTT	1037–1058	courtesy Dr. Mark Hahn
3RACE1	F	TTCCACGGCCGTTTGAAACTCCTCCAT	1200–1227; 2513–2540	
AHR1	F	ATCATGTGAAAAATTATGAAAACC	1250–1273	
AHR2	R	TTACTTGTGATCCAAAAAGATTC	2685–2708	
3RACE2	F	AGCGGAGCCTGCAGCTACCTTTTCA	1492–1517	
3RACE2nest	F	GTCCGCCCGGGTTCCTGGACTACTT	1492–1517	
3RACE3	F	GCGGAGCCTGCAGCTACCTTTTCACTT	1580–1607	
3RACE3nest	F	CAACTCGCTCCTAGGGGCCATGATGAA	1580–1607	
5RACE1	R	GGAGCAGCATGTCCCTTCGGAGAA	239–264	
AHR3	F	AGAGCCAGGAAATGGATGAC	1667–1687	
AHR4	R	AGCTTGCACTGGAGACACCT	2630–2650	
BFA5'2	R	TTCTCGGGGCAGAGGTGCTGGGGGCTAG	1_28	Yasui et al. (2004)
AHR5	F	GGGTTTCCCTTGGGAGTCT	1_19	
AHR6	R	TTCGTCTTGGTTCATCATGG	1685–1705	
AHR7	F	AGCCTGCAGCTACCTTTTCA	1458–1478	
AHR8	R	AAAGGAATGGCTTCCAGACA	2705–2725	
3RACE4	F	TGCCACTCCAAATCAGCTGGGAATCA	2463–2489	
3RACE5	F	CTGGCCACCTGTCTGGAAGCCATTC	2952–2977	
seq1	F	GATTGTAGGATACCTGAAAGTGA	531–554	
seq2	F	TTTCATCAGTCAGACATCATCTA	769–792	
seq3	F	ATCATGTGAAAAATTATGAAAACC	1337–1360	
seq4	F	GCCTGCAGCTACCTTTTCA	1408–1428	
seq5	F	AGCCTGCAGCTACCTTTTCA	1458–1478	
seq6	F	ACAATTTTCATACAGGACTGCT	1582–1604	
seq7	F	GAACCAAGACGAATCCATATAC	1693–1715	
seq8	R	GTGGGAACCCAGATTCTTT	1927–1947	
seq9	F	AAAAGATAATGGAAAGAATCTGG	2036–2059	
seq10	R	TTTAAACAATATCTCCTCCACAC	2013–2036	
seq12	R	CCAGATATTTTCATTCTGTAGAGC	2873–2896	
seq13	F	AGGCCATGAAATCAGTCCAC	3403–3423	
seq14	R	GGAGCAGGTCTTTTCCTTC	3362–3378	
q181F	F	ATTCTCTGTCCGTTCCTCC	181–202	
q314R	R	TCGATAAGCTTTCCGTCTCC	292–314	
ACTIN				
β-actin F	F	CAGGTCATCACCATYGGCAA	NA	Valenzuela and Shikano (2007)
β-actin R	R	GCTTGCTGATCCACATCTGC	NA	Valenzuela and Shikano (2007)

Oligo/Direction		Sequence (5' to 3')	Position	Reference
actin F	F	TCGAAAGGACCTGTATGCCAA	NA	
actin R	R	TAGGTGCCAGAGCGGTGATT	NA	

Table 2

Primers used in cloning the red-eared slider AHR cDNA sequences. Primers are listed in the chronological order of use in cloning. Forward primer (F), reverse primer (R), sequencing primer (seq), rapid amplification of cDNA ends (RACE), nested primer (nest), quantitative PCR primers (q).

Oligo	Direction	Sequence (5' to 3')	
seq1	F	TTATTGTGCTGAGAATCATATCC	
seq2	R	CTTTCTTGTCTGTCCATGAAG	
seq3	F	TTGATTCATACAGAAGACAGACC	
seq4	R	GATCTAACTCTGCATTCAATCTG	
PS5RACE1	R	TGCCAATGAAGCTGACGCTGAAAC	
PS5RACE1nest	R	GGAACCCAGATAGTCCTGGATGGTTGA	
PS3RACE1	F	TGGACTTTACCCCGTGGCCTGTGA	
PS3RACE1nest	F	TCCGAATGATAAAGACCGGCGAAAGC	
qPSAHR1-66F	F	TGCCTTTCCACAAGATGTTATC	
qPSAHR1-150R	R	AAGCTTTTGGCTCTCAGGTAATC	
PP1F	F	CAGCAGACCCTGAGAACTTCTTCCTG	Ramsey et al. (2007)
PP1R	R	GCGCCTCTTGCACTCATCAT	Ramsey et al. (2007)