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Cloning of estrogen receptor alpha and aromatase cDNAs 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 (*Chrysemys picta*) 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 playing a significant role in reproduction are affected. cDNA sequences were determined for estrogen receptor alpha and aromatase in the painted turtle. These genes were measured in our study animals using quantitative PCR. Adult turtles were trapped from our study site (Moody Pond, MP) or a reference site (Washburn Pond) and exposed to laboratory environments containing soil from either site. The red-eared slider (*Pseudemys scripta*), a pond turtle closely related to the painted turtle, was used to assess neonatal exposure to soil and water from the sites. Our results show an increase in hepatic estrogen receptor, which suggests exposure to estrogenic contaminants. Female turtles from MP appear to have a long-term effect on hepatic ER. Other findings were apparent age-dependent differences in expression of aromatase and ER in the brains of neonate and year-old juvenile turtles. Phylogenetic analyses of the cDNA sequences further support the hypothesis that turtles are in a sister clade to birds and crocodilians.

1. Introduction

1.1. Background

Reproductive changes, including reduced plasma estradiol and VTG, have been described in female painted turtles from Moody Pond. It is likely that exposure to xenoestrogens, antiestrogens, antiandrogens, or other EDC contributed to these changes (Rie et al., 2005). Females have been observed to have reduced ovarian follicular reserve (Rie et al., 2005). In males of the same species from the contaminated site at Moody Pond, 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 p4501A1, suggesting contamination of Moody Pond with organic compounds such as xenoestrogens or dioxin-like compounds (Rie et al., 2000). These studies suggest that both male and female turtles are sensitive to EDC present at this site.

Effects of the Moody Pond environment have also been studied in zebrafish (*Danio rerio*). Zebrafish embryos exposed to Moody Pond sediment elutriate during development showed an increase in aromatase B (aromB), as well as induction of a novel splice variant using semiquantitative PCR (Novillo-Villajos et al., 2003). When zebrafish embryos were treated with 0.1 μM E_2, the resultant expression pattern was the same. This suggests that the contaminant(s) in Moody Pond sediment may act as environmental estrogens. In the same study, to investigate induction of the novel aromB splice variant, zebrafish embryos were exposed to 0.5 nM dioxin from 6 to 48 h post fertilization or 0.1 μM E_2 from 2 to 48 h post fertilization. Amounts of the splice variant were relatively increased with exposure to dioxin and were decreased with E_2 treatment. These results suggested that induction of the splice variant was likely due to the presence of dioxin-like contaminants in Moody Pond sediment. Moody Pond water alone did not have an effect.

The Massachusetts Military Reservation (MMR) is a military training site on Cape Cod that was declared an Environmental Protection Agency Superfund site in 1989. The study site is located very close to sources of contamination from the MMR (see Materials and methods for detail) and the reference site is to the east of the MMR and away from the contaminated plumes, which flow to the southeast.

1.2. Estrogen receptor

A key element in the signal transduction pathway for EDC impact is the estrogen receptor (ER). Assessing changes in ER expression in normal and EDC-exposed animals is critical to our understanding of the role of these receptors in endocrine disruption in the natural environment. Estradiol (E_2) is known to cause an increase in hepatic

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ER, plasma vitellogenin and vitellogenin mRNA in female and male turtles (Custodia-Lora et al., 2004a, 2004b).

The likely ancestral condition for jawed vertebrates is the presence of two ER isoforms: ER alpha (ERα) and ER beta (ERβ). Both ER isoforms have been found in fish, mammals, amphibians, birds, and reptiles, including the American alligator (*Alligator mississippiensis*) and the red-belly slider (*Pseudemys nelsoni*) (Katsu et al., 2004, 2008). ERα has been identified in the Nile crocodile (*Crocodylus niloticus*) and a partial sequence in the painted turtle (Custodia-Lora et al., 2004b; Katsu et al., 2006). ER sequences have been obtained from the caiman (*Caiman crocodilus*), whiptail lizard (*Cnemidophorus uniparens*), red-eared slider turtle, and in a lizard, the anole (*Anolis carolinensis*) (Young et al., 1995; Bergeron et al., 1998; Sumida et al., 2001; Custodia-Lora and Callard, 2002). Katsu et al. (2004) showed that ERα alone was sensitive to estradiol exposure in juvenile alligators, while ERβ and progesterone receptor were not responsive to estradiol exposure. For the purposes of this study, we focused on ERα mRNA.

Since estrogen is known to have a role in regulating ER in the painted turtle, changes in ER levels may be linked to estrogen production itself and thus aromatase is a gene of interest (Giannoukos and Callard, 1996).

1.3. Aromatase

The enzyme aromatase (CYP19 P450 aromatase) converts C_{19} steroids such as testosterone and androstenedione to estrogen. The enzyme is located primarily in the brain and ovary among vertebrates, but is also expressed in the placenta of primates and artiodactyls, and in the placenta, adipose, and bone in humans (Conley and Hinshelwood, 2001). Aromatase cDNAs have been cloned in the red-eared slider turtle (*Pseudemys scripta*) and the leopard gecko (*Eublepharis macularius*) (Murdock and Wibbels, 2003; Endo and Park, 2005).

Brain aromatase and estrogen are closely linked to reproductive behaviors in vertebrates (Wu et al., 2009). In a study by G.V. Callard et al. (1977), aromatase was first identified and quantified by measuring conversion of androgen to estrogen in painted turtle brain and ovary. *CYP19* expression in the adrenal–gonad–kidney (AKG) complex was examined during several early developmental stages by *in situ* hybridization and quantitative PCR and the pattern of expression found to be similar in painted turtle and softshell turtle (Valenzuela and Shikano, 2007).

A number of studies on turtle sex determination have focused on aromatase activity during embryonic development. Ramsey and Crews (2007a, 2007b) determined that gonadal aromatase gene expression could not be predicted from measurements of the AKG, as measurements of gonad alone yielded different results. In this study the gonad was isolated from the AKG complex for analysis.

The role that aromatase and estrogens play in control of sex determination, differentiation, and in adult follicular selection for further development and vitellogenesis is unclear. Since endocrine disruption has been identified as a major problem of aquatic wildlife, it is important to identify, characterize, and quantify genes involved in reproduction, such as the ER and aromatase. In this study, the cDNA sequence of painted turtle *ERα* and *CYP19* was determined, and gene expression was measured in both painted turtles and red-eared sliders exposed for 1 year to a test site (Moody Pond) and reference site (Washburn Pond) on Cape Cod, MA, USA.

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 m 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 the sites in Mashpee, MA for a 13-month laboratory exposure to sediment from the study 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 was placed in the Washburn environment tanks; also a subset of Washburn Pond individuals was 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 males 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.

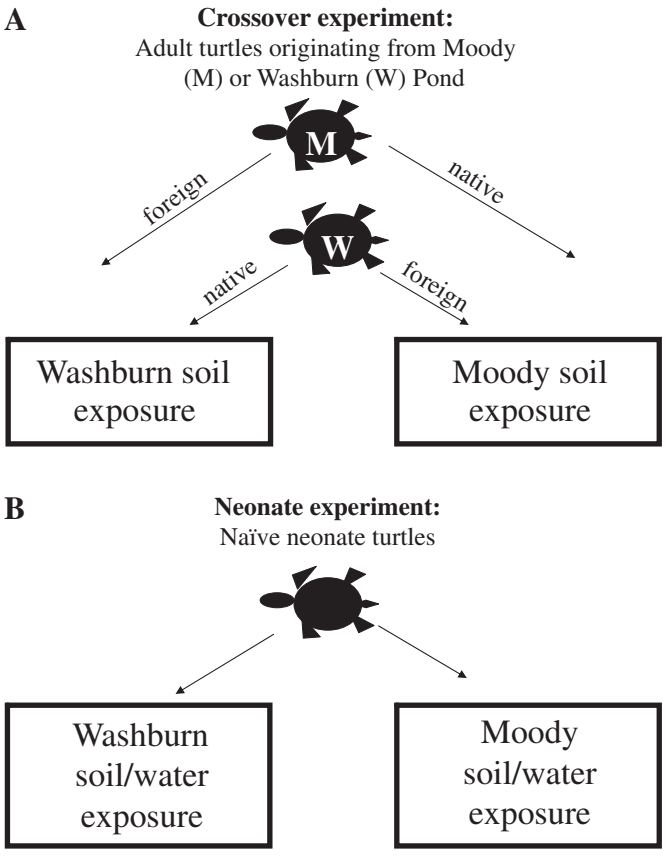

Fig. 1. Diagram of experimental design. (A) Adult painted turtles trapped from Moody Pond or the reference site Washburn Pond were exposed to mimicked environments representing either their native environment or the foreign environment in the laboratory. The purpose of the "crossover" was to determine 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.