Effects of Embryonic and Adult Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Hepatic Microsomal Testosterone Hydroxylase Activities in Great Blue Herons (*Ardea herodias*)

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Introduction

Great blue herons (*Ardea herodias*) in south-west British Columbia have been used by the Canadian Wildlife Service as sentinels of environmental contamination with halogenated aromatic hydrocarbons (HAHs) since the 1970s. Great blue herons have been observed to suffer from reproductive problems and other adverse effects in areas that were contaminated with relatively great concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls[1-4]. Hepatic microsomal CYP1A-associated ethoxyresorufin O-deethylase (EROD) activity was found to be a sensitive and reliable biomarker of exposure and potential toxicity of HAH-contamination in great blue heron embryos[4].

In our continued effort to identify biomarkers of exposure of herons to HAHs, and to examine alterations in biochemical parameters that are relevant to reproductive capacity, we examined the effects of TCDD on hepatic microsomal testosterone hydroxylases. In the liver, testosterone hydroxylations are performed by the cytochrome P-450 (CYP450) family of enzymes. Sex and developmental differences exist in the CYP450-dependent metabolism of steroids and these differences are controlled by hormones[5-7]. It is known that increased androgen secretions during the perinatal period in male rats are responsible for male sexual development after puberty, and this "masculinization" is reflected in the expression of male-specific and repression of female-specific CYP450 enzymes. Disruptions in the 'normal' hormonal secretion patterns during this critical period can, therefore, have consequences later on in puberty or adulthood, and may be reflected in changes in the activities of sex-specific CYP450s.

TCDD and related chemicals elicit antiestrogenic and antiandrogenic responses in laboratory animals[8-10]. TCDD exposure during the perinatal period (*in utero and lactational*) has been reported to alter androgenic status in male rats that lasted into adulthood and led to a reduced reproductive capacity[11-13]. We suggest that embryonic (*in ovo*) and adult TCDD exposure in wild avian species can cause alterations in hormonal status as well, and that these changes are, in part, responsible for reduced reproductive capacity. In the present study, we specifically hypothesize that disruptions of hormonal status by TCDD exposure may be reflected in changes in the activities of hepatic microsomal steroid metabolizing CYP450 enzymes. The objectives were two fold: 1) Characterize the hepatic microsomal testosterone hydroxylase activities of unexposed herons at various ages. 2) Evaluate the effect of TCDD exposure during embryonic development and adulthood on these activities.

Methods

Birds and Treatments: Thirteen adult great blue herons, raised from hatchling stage at the University of British Columbia were injected intraperitoneally with corn oil (5 males and 2 females) or 20 μg TCDD/kg in corn oil (4 males and 2 females)[14]. These birds were intended for the establishment of a permanent captive breeding colony, but this project could no longer be maintained due to discontinuation of funding. The birds were sacrificed two weeks after dosing. Developing heron eggs were collected from a relatively uncontaminated colony and injected via the air sac with corn oil or 2 μg TCDD/kg, about half-way through their full-term 28-day incubation[15,16]. The birds were sacrificed either at hatch or fed an uncontaminated diet for 7 days post-hatch. Liver microsomes were prepared immediately after sacrifice.

Testosterone Hydroxylase Assay: Hydroxylated metabolites of testosterone were analyzed by HPLC (Waters, Millipore, Milford, MA) using a modification of the method of Wood *et al.*[17] Metabolites were detected using a UV detector (Waters model 484) at 254 nm. Metabolites were identified on the basis of retention time and quantified using authentic standard curves. Reaction times were 15 or 20 min dependent on the activity of the sample. The steroid 5α-reductase inhibitor, 4-MA, used to prevent the reduction of testosterone in female rat microsomes, had no effect (at a concentration of 2.5 μM) on the activities in adult heron liver microsomes of both sexes, and was not used in the rest of the study.

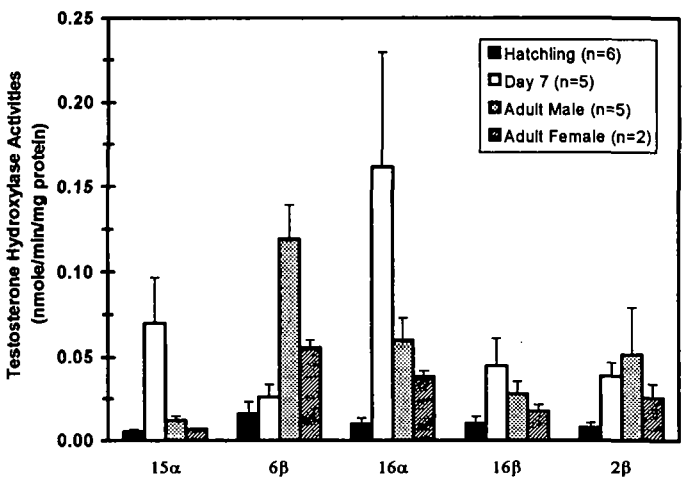

Figure 1. *Hepatic microsomal testosterone hydroxylase activities (nmole/min/mg protein) in untreated great blue herons at various ages. Each sample was assayed in duplicate. Error bars represent standard deviations. The five activities shown were the only ones found in the present study, with the exception of an additional unidentified metabolite formed by 7-day old herons. The sex was not determined in the hatchlings and 7-day old herons.*

Results

Influence of Age: Untreated great blue heron liver microsomes hydroxylated testosterone at the 2β, 6β, 15α, 16α and 16β position, but not at the 1β, 2α, 7α, 11β, 15β, or 19 position. The formation of these metabolites was NADPH- and time-dependent. In 7-day old great blue herons, an additional unidentified metabolite was observed in the chromatograms of samples separated on the Zorbax column, which did not match any of the metabolites for which authentic standards were available. Testosterone hydroxylase activities were strongly influenced by the age of the birds (Fig. 1). Activities

were lowest in the hatchlings. In 7-day old chicks activities were considerably higher than in the hatchlings. In the adult birds, on the other hand, 6β-testosterone hydroxylase activity was increased relative to the younger birds, while 15α- and 16α-testosterone hydroxylase activities were considerably lower than in the 7-day olds, although somewhat higher than in the hatchlings.

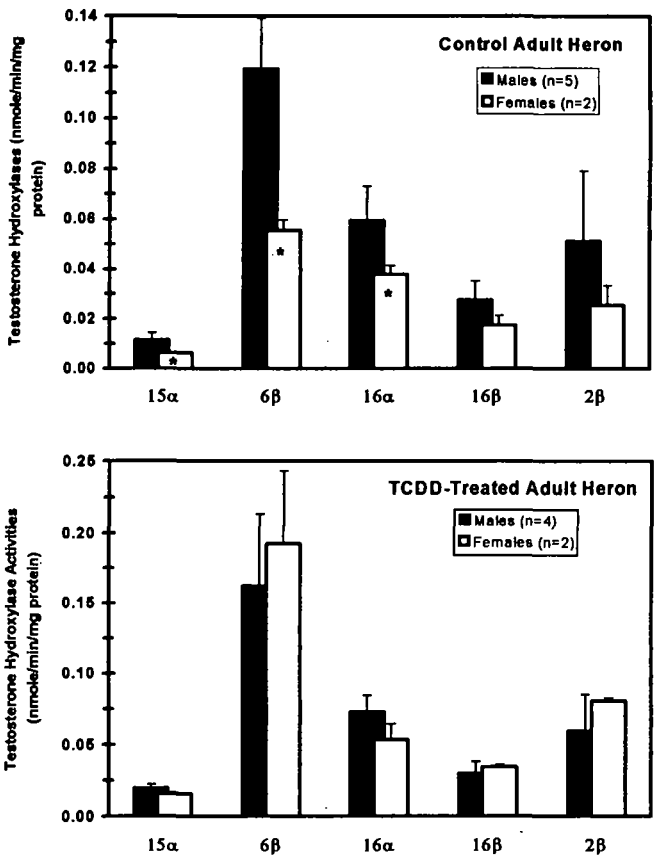

Figure 2. *Hepatic microsomal testosterone hydroxylase activities in adult great blue herons of both sexes; control (top) and TCDD-treated birds (bottom). * - Significantly lower in female than in male, using one-way ANOVA ($p<0.05$).*

Effect of TCDD: In untreated adult herons, 15α-, 6β-, and 16α-testosterone hydroxylase activities were significantly lower in female than in male liver microsomes (Fig. 2, top). After treatment with 20 μg TCDD/kg, these differences disappeared (Fig. 2, bottom). In the females, all hydroxylase activities, except for 16α-, were significantly ($p<0.05$) greater after TCDD treatment; in the males only 15α-testosterone hydroxylase activity was significantly greater after treatment. In the hatchlings, TCDD treatment *in ovo* resulted in a significant increase in hepatic microsomal 6β-testosterone hydroxylase activity. None of the other activities were significantly affected. In the 7-day old herons, TCDD treatment *in ovo* did not cause any significant changes in the enzyme activities. In the 7-day old birds the induction of 6β-testosterone hydroxylase activity was probably no longer apparent due to the decreased body burden of TCDD relative to the hatchlings. TCDD concentrations (ng/g wet weight) in livers were 11.3 ± 0.84 for the hatchling and 0.82 ± 0.11 for the 7-day old herons[16].

Comparison of 6β-Testosterone Hydroxylase with EROD Activity: Hepatic EROD and 6β-testosterone hydroxylase activities of individual microsomal preparations were significantly ($p<0.05$) correlated in hatchlings ($r=0.91$; $n=12$; Fig. 3, *left*) and female adult herons ($r=1.0$; $n=4$) (Fig 3, *right*), but not in the 7-day-old chicks and male adult herons.

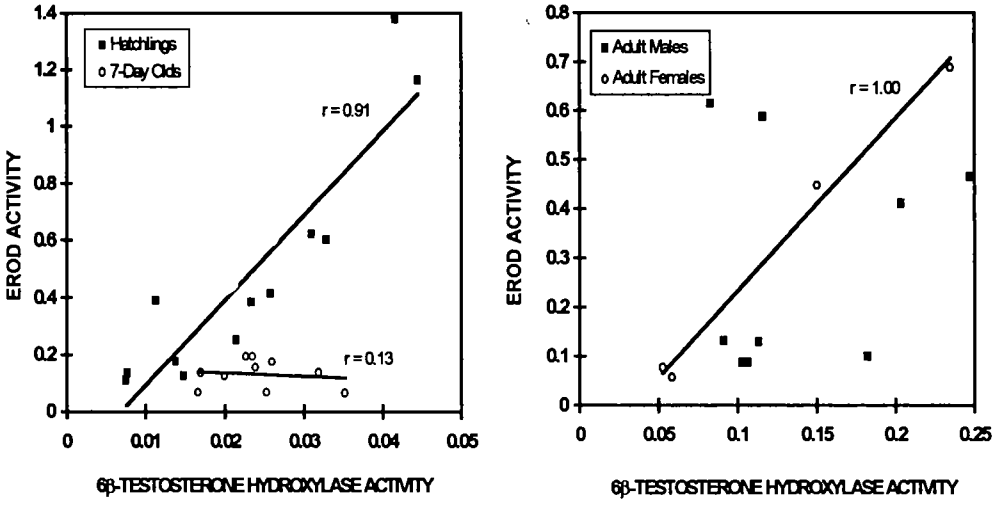

Figure 3. *Comparison of hepatic microsomal 6β-testosterone hydroxylase and ethoxyresorufin O-deethylase (EROD) activity (nmole/min/mg protein) in control and TCDD-treated herons of various ages.*

Discussion

Comparison with Rat Testosterone Hydroxylases: The profile of hepatic microsomal testosterone hydroxylase activities in the untreated herons is different from that observed in rats[17,18]. In both species, 6β-hydroxytestosterone is one of the major metabolites formed, but in rats (and other rodents) several other hydroxylated metabolites are found beside the five formed by the heron microsomes in the present study. The sex-specific enzyme profiles of testosterone hydroxylase activities that have been well characterized in rats[19-23], and the effects of 3-methylcholanthrene (3-MC)-type inducers thereon[17,24,26], are not consistent with those observed in the control and treated adult herons. It is likely that the CYP450s involved in the hydroxylation of testosterone in heron liver microsomes are either of a different nature or expressed and regulated differently from those in rat.

Comparison with Chickens: In liver microsomes of adult chickens 6β-, 16α- and 2α-testosterone hydroxylase activities have been observed to be sexually dimorphic, with activities consistently lower in females than in males[25]. 6β- and 16α-testosterone hydroxylase activities in male and female chickens were comparable to those in our herons and we observed a similar sexual dimorphism, although herons did not demonstrate any 2α-testosterone hydroxylase activity.

Relationship between 6β-Testosterone Hydroxylase and EROD Activity: It is known that in uninduced rats, hepatic microsomal 6β-testosterone hydroxylation is catalyzed to a small degree by CYP1A, although mainly by CYP3A isoenzymes. In rats, CYP3A hydroxylates testosterone at the 1β and 15β positions, activities which were not detected in our herons. 3-MC-type inducers decrease 6β-testosterone hydroxylase activity rats[6,17,26], presumably because of the simultaneous induction of CYP1A and inhibition of CYP3A. In contrast, in the present study, 6β-testosterone hydroxylase

activities were significantly induced by TCDD in the female adult and the hatchling herons, although not in the 7-day old herons. The presence of a TCDD-inducible analogue of rat CYP1A1 has been demonstrated in the great blue heron[27]. We observed strong correlations between 6β-testosterone hydroxylase and CYP1A-associated EROD activity in individual microsomal preparations of heron hatchlings and female adults (Fig. 3). EROD activity did not correlate with any of the other hydroxylases measured. We suggest that in the case of the heron hatchling and adult female, CYP1A is responsible for a significant proportion of the metabolism of testosterone to its 6β-hydroxylated metabolite. It is further likely that other CYP450 enzymes contribute to this reaction and that it is possible that those unidentified enzymes are present in the 7-day old herons and male adults at greater levels than in the hatchlings and female adults, confounding a clear relationship between EROD and 6β-testosterone hydroxylase activity. In support, it can be seen that basal 6β-testosterone hydroxylase activities (but not EROD) are indeed higher in the 7-day old herons than the hatchlings and higher in the male than the female adults (Fig. 1 and 3).

Effects of TCDD: The observation that sex differences in hepatic microsomal testosterone hydroxylases in the adult great blue herons disappeared after treatment with TCDD (Fig. 2), indicates a 'defeminization' of the female enzyme pattern. Considering the small sample size, however, this finding should be considered preliminary. It is not known if this defeminization in the females is caused indirectly by TCDD-mediated alterations in hormonal status or due to direct induction of enzymes, but the latter is more likely. In a parallel study, no significant changes in hormonal status of the herons (chicks and adults) were observed after TCDD exposure[14-16]. It is not known at present whether the observed alterations in hepatic microsomal testosterone hydroxylase activities in the female herons can result in a reduced reproductive capacity, but it is possible that these changes, if persistent, interfere with the metabolism and physiological function of steröid hormones in the long-term.

Future studies are required to investigate whether alterations in hepatic testosterone hydroxylases in avian species can lead to reduced reproductive capacity. It is also not known whether exposures to TCDD during the embryonic stage in these birds can interfere with sexual imprinting and result in altered expression of these activities and other sexual characteristics in adulthood. Many basic studies are still required to characterize avian cytochrome P-450 enzymes, their associated catalytic activities, their (sex-specific) expression and regulation, and the effects of environmental contaminants.

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