EFFECTS OF ACUTE 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN EXPOSURE ON PLASMA THYROID AND SEX STEROID HORMONE CONCENTRATIONS AND ESTROGEN RECEPTOR LEVELS IN ADULT GREAT BLUE HERONS

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Abstract—Adult great blue herons (Ardea herodias) were exposed intraperitoneally to a single dose of 20 μg/kg body weight of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and sacrificed after 14 d. Approximately 1.9% of the total TCDD dose was measured in the liver. No differences were found in liver weight or liver to body weight ratio between control and TCDD-treated birds. Hepatic microsomal ethoxyresorufin O-deethylase (EROD) activity was induced sixfold above control activities in TCDD-exposed herons (p < 0.001). Plasma total T4 concentration was significantly elevated in TCDD-exposed herons (p < 0.05). The TCDD treatment had no effect on plasma total T3 levels, or on the plasma T3 to T4 ratio. No effect of this TCDD exposure was found on plasma 17β-estradiol or testosterone concentrations, or on hepatic estrogen receptor affinities and concentrations in male or female herons.

Keywords—Great blue heron 2,3,7,8-Tetrachlorodibenzo-p-dioxin Thyroid hormones Sex steroid hormones Ethoxyresorufin O-deethylase

INTRODUCTION

Great blue heron (Ardea herodias) colonies in coastal and estuarine areas of the Strait of Georgia in southwestern British Columbia, Canada, have been monitored by the Canadian Wildlife Service for two decades. Levels of persistent chlorinated hydrocarbons have been measured in heron eggs since 1977, and reproductive success has been monitored in several colonies since 1983. Great blue herons provide a good indication of local environmental quality because they occupy a high trophic level and are nonmigratory in this area [1]. Concentrations of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) in heron eggs collected in the early 1980s were found to be unusually high in certain colonies and to vary considerably between colonies [1]. The Strait of Georgia contains a large number of Kraft pulp and paper mills that until recently used molecular chlorine as a bleaching agent. Effluent from mills employing the Kraft bleaching process is known to contain trace quantities of PCDDs and PCDFs [2]. Levels of PCDDs and PCDFs in biota occurring near several coastal mills have exceeded federal health guidelines for safe consumption, resulting in fisheries closures.

In 1987, a heron colony near a pulp and paper mill in the Strait of Georgia failed to fledge a single hatching out of 57 active nests. This reproductive failure coincided with significantly elevated concentrations of PCDDs and PCDFs in heron eggs, particularly 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as compared with the previous year (mean 210 ng TCDD/kg egg, wet weight, n = 10 [1]). In 1988, studies in hatchlings found several developmental toxicities correlated with TCDD levels, including subcutaneous edema, decreased body and skeletal growth, decreased plasma calcium concentration, and reduced number of down follicles [3]. Hepatic microsomal ethoxyresorufin O-deethylase (EROD) activity was shown to be a highly sensitive and specific biochemical marker of exposure to TCDD in herons [4–6]. Further laboratory studies in heron hatchlings exposed in ovo to TCDD examined a number of hormonal endpoints as potential markers of TCDD toxicity, including plasma thyroid and sex steroid hormone concentrations and hepatic estrogen receptor levels, and found these responses to be less sensitive to TCDD exposure than EROD activity, body weight loss, and embryo lethality [7,8]. Overall, these studies concluded that certain areas in the Strait of Georgia had accumulated environmental concentrations of TCDD and related chemicals that were beginning to cause toxicities in herons [9]. Remedial process changes in local pulp and paper mills have greatly lowered the release of PCDDs and PCDFs into the environment, and concentrations of these chemicals in heron eggs have declined rapidly [10].

Most ecotoxicological studies in piscivorous birds have focused on juveniles and little information is available on biochemical responses of adult birds exposed to persistent contaminants such as TCDD (see [11,12] for reviews). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons (HAHs) have been shown to modulate a number of endocrine systems in vertebrates, including thyroid hormones and sex steroid hormones and their receptors [13]. Alterations in endocrine homeostasis during adulthood in wild avian species such as the great blue heron may have adverse effects on reproductive capacity. The purpose of this study was to examine the effects of acute TCDD exposure on plasma sex steroid and thyroid hormone concentrations and hepatic estrogen receptor levels in adult great blue herons.

MATERIALS AND METHODS

Chemicals

Tritiated 2,3,7,8-TCDD ([3H]TCDD; 40 Ci/mmol; 97% radiochemical purity) and nonradioactive TCDD were purchased...
of Burke and Mayer [15], optimized for avian samples in our laboratory [16]. Protein concentrations in microsomal and cytosolic preparations were determined using the method of Bradford [17].

Hepatic estrogen receptor (ER) affinities ($K_d$) and concentrations ($B_{max}$) were measured in cytosols using saturation analysis [18], with modifications described previously [8]. The ER assays were performed on the same day as cytosol preparations to avoid repeated freeze–thawing of liver samples. The $[\text{3H}]$estradiol concentration range used for the assay was 0.5 to 12 nM. Nonspecific binding was determined in the presence of 100-fold molar excess of diethylstilbestrol.

Hormone determinations were performed by radioimmunoassay (RIA) using commercially available $[\text{125I}]$ kits (ICN Biomedicals, Costa Mesa, CA, USA), with modifications [7, 8]. Plasma total T$_3$, total T$_4$, and 17$\beta$-estradiol concentrations were measured in unextracted plasma. Plasma testosterone concentrations were measured in diethyl ether-extracted plasma samples as described previously [8]. Several procedures were performed to determine the performance characteristics of each RIA. Intra- and interassay variation were determined using domestic chicken plasma samples for total T$_3$, total T$_4$, and estradiol, and commercially available sera of human origin for testosterone. Intraassay coefficients of variation (CVs; $n = 6$) were 4.6%, 3.7%, 8.1%, and 4.4%, and interassay CVs ($n = 12$) were 5.7%, 5.4%, 10.5%, and 7.1% for T$_3$, T$_4$, estradiol, and testosterone, respectively. Serial dilutions of heron plasma were assayed and compared to the commercial (human origin) hormone standard curves supplied with each RIA kit. Parallelism between dilutions of adult heron plasma and hormone standards was demonstrated for each RIA as determined by analysis of covariance (i.e., homogeneity of linear regressions) [19]. The lower limits of detection (pg/ml), defined as two SDs of the binding observed with the hormone-free (zero) standard, were 2.3, 430, 1.1, and 1.9 for total T$_3$, total T$_4$, estradiol, and testosterone, respectively.

**Analyses of $[\text{3H}]$TCDD**

Concentrations of $[\text{3H}]$TCDD were measured in liver, kidney, breast muscle, and fat of herons using hexane extraction of tissue homogenates followed by liquid scintillation counting [6]. Efficiency of the extraction procedure ranged from 81 to 84% and was determined from untreated tissues spiked with $[\text{3H}]$TCDD as described previously [6]. Quenching ranged from 2 to 7% in liver and kidney, 15 to 27% in fat, and 13 to 22% in muscle. In each sample, quenching was corrected for using the internal standard method. The $[\text{3H}]$TCDD concentrations were expressed based on tissue wet weight; lipid-corrected concentrations were not determined. The percentage of TCDD present in liver and kidney was calculated as the mass of TCDD in each whole organ divided by the total mass of TCDD injected.

**Statistical analyses**

Apparent estrogen receptor affinities ($K_d$) and concentrations ($B_{max}$) were determined using Woolf plot analysis [20]. Least-squares linear regression was performed on Woolf plots to determine $K_d$ and $B_{max}$ values for individual samples. Statistically significant differences between control and TCDD-treated herons were detected using one-way analysis of variance. Statistical significance was set at $p < 0.05$. 

**Sample preparation**

On the day of sacrifice, herons were weighed and blood was drawn from wing veins and collected in heparinized glass test tubes, on ice. Birds were decapitated, the body cavity was opened, and the gall bladder was tied off with surgical thread. These birds served as controls and were injected intraperitoneally (i.p.) with 1 ml of corn oil. Six herons (2 females, 5 males; 2,460 g) were located at an aviary at San Rafael Research Aviary near White Rock, British Columbia, Canada. These birds were served as controls and were injected intraperitoneally (i.p.) with 1 ml of corn oil. Six herons (2 females, 5 males; 2,460 ± 93 g) were located at the UBC San Rafael Research Aviary near White Rock, British Columbia, Canada. These birds served as controls and were injected intraperitoneally (i.p.) with 1 ml of corn oil. Six herons (2 females, 5 males; 2,460 ± 90 g) were located at an aviary at UBC and were injected i.p. with 20 μg/kg body weight of $[\text{3H}]$TCDD dissolved in corn oil. Birds at both aviaries were held outdoors under natural photoperiod. Herons were fed a diet of whole herring (Clupea harengus) and rainbow trout (Oncorhynchus mykiss) for the 14 d prior to sacrifice.

**Hepatic EROD activity was determined in microsomes prepared from individual birds using the direct fluorometric assay of Burke and Mayer [15], optimized for avian samples in our laboratory [16]. Protein concentrations in microsomal and cytosolic preparations were determined using the method of Bradford [17].

**Hepatic EROD activity was determined in microsomes prepared from individual birds using the direct fluorometric assay**

**Animals and treatments**

Great blue heron eggs were collected from several breeding colonies in southwestern British Columbia in April 1990 and 1991. Fertile eggs were artificially incubated and hatchlings were raised at the Department of Animal Science, University of British Columbia (UBC), Vancouver, B.C., Canada [14]. A total of 13 herons (4 females and 9 males) were raised with the intention of creating a captive breeding colony. Our original objective was to conduct a chronic study in breeding herons exposed to HAHS via food and examine effects in their offspring. However, breeding was unsuccessful after 3 to 4 years and it was not economically feasible to continue maintaining the heron colony. Because the herons could not be released back to their natural setting, an acute (14-d) TCDD exposure study was conducted in February 1994. Seven herons (2 females, 5 males; 2,460 ± 93 g) were located at the UBC San Rafael Research Aviary near White Rock, British Columbia, Canada. These birds served as controls and were injected intraperitoneally (i.p.) with 1 ml of corn oil. Six herons (2 females, 4 males; 2,688 ± 90 g) were located at an aviary at UBC and were injected i.p. with 20 μg/kg body weight of $[\text{3H}]$TCDD dissolved in corn oil. Birds at both aviaries were held outdoors under natural photoperiod. Herons were fed a diet of whole herring (Clupea harengus) and rainbow trout (Oncorhynchus mykiss) for the 14 d prior to sacrifice.

**Sample preparation**

On the day of sacrifice, herons were weighed and blood was drawn from wing veins and collected in heparinized glass test tubes, on ice. Birds were decapitated, the body cavity was opened, and the gall bladder was tied off with surgical thread. The liver was perfused via the hepatic portal vein with ice-cold Tris/KCl buffer (0.05 M Tris, 1.15% KCl, pH 7.5). The liver was removed and weighed, and approximately 5 g was placed in a beaker containing 20 ml of ice-cold Tris/KCl buffer for microsomal preparation. Other liver samples were immediately frozen in liquid nitrogen for determination of estrogen receptor levels and $[\text{3H}]$TCDD concentrations. Kidneys, gonads, thyroid glands, and spleen were removed and weighed. Samples of breast muscle and adipose tissue were removed and frozen for determination of $[\text{3H}]$TCDD concentrations. Plasma was prepared by centrifugation and stored at −20°C. Hepatic microsomes were prepared as described previously and stored at −80°C [4]. Hepatic cytosol was prepared using HEDGM buffer (25 mM N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid [HEPES], 1.5 mM ethylenediaminetetraacetic acid [EDTA], 1.0 mM dithiothreitol, 20 mM sodium molybdate, 10% v/v glycerol, pH 7.6) as described previously [6].

**Biochemical assays**

Hepatic EROD activity was determined in microsomes prepared from individual birds using the direct fluorometric assay of Burke and Mayer [15], optimized for avian samples in our laboratory [16]. Protein concentrations in microsomal and cytosolic preparations were determined using the method of Bradford [17].
Table 1. Hepatic ethoxyresorufin O-deethylase (EROD) activities, plasma total T4 and total T3 concentrations, and plasma T3 to T4 ratios in adult great blue herons after 14 d of exposure to a single intraperitoneal injection of 20 µg/kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or corn oil vehicle (± SE).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic EROD activity (pmol/min/mg protein)</td>
<td>n</td>
</tr>
<tr>
<td>Plasma total T4 (ng/ml)</td>
<td>7</td>
</tr>
<tr>
<td>Plasma total T3 (ng/ml)</td>
<td>7</td>
</tr>
<tr>
<td>Plasma T3 to T4 ratio</td>
<td>7</td>
</tr>
<tr>
<td>Plasma T4 to T3 ratio</td>
<td>7</td>
</tr>
</tbody>
</table>

*Significantly different from control by analysis of variance (ANOVA) (p < 0.001).
* *Significantly different from control by ANOVA (p < 0.05).

**RESULTS AND DISCUSSION**

No differences were found in liver weights or liver to body weight ratios between control and TCDD-exposed herons (data not shown). Hepatic EROD was induced approximately sixfold in TCDD-exposed herons (p < 0.001; Table 1). This level of EROD induction suggests a moderate TCDD dose, although there is no information on maximal EROD activity in adults of this species. However, EROD induction of 20- to 40-fold or greater above control activities is common in the large number of vertebrate species studied to date. Uninduced (control) EROD activities in adult herons were similar to those measured in 7-d-old herons (92 ± 16 pmol/min/mg protein) in a previous study [7].

Many of the biological effects of TCDD exposure are mediated via the aryl hydrocarbon (Ah) receptor, and induction of cytochrome P450 1A1-associated monooxygenases such as EROD is the best characterized response under direct regulation of the Ah receptor [21]. Our laboratory has validated the use of hepatic EROD activity in great blue herons as a biochemical marker of environmental exposure to TCDD. Monospecific antibodies raised against rat cytochrome P450 1A1 recognized a protein in hepatic microsomes of the heron, providing evidence that EROD activity in the heron is due to cytochrome P450 1A1 [6]. Hepatic cytosols prepared from adult and juvenile herons had Ah receptor binding affinities that were of the same order of magnitude as that reported for human placenta [6]. Furthermore, process changes in pulp mills in the Strait of Georgia that resulted in decreased PCDD/PCDF contamination were reflected in decreased induction of EROD activity in heron hatchlings [5].

Plasma total T4 concentration was significantly elevated in TCDD-treated herons (p < 0.05). However, there was no significant effect of TCDD exposure on plasma total T3 concentration or the plasma T3 to T4 ratio, although the plasma T3 to T4 ratio appeared lower (p = 0.074) in TCDD-exposed birds (Table 1). Because T3 is the physiologically active thyroid hormone [22] and is metabolized from T4, the plasma T3 to T4 ratio is used as a measure of the relative levels of circulating thyroid hormones. Thyroid gland weights did not differ between control and TCDD-treated herons.

2,3,7,8-Tetrachlorodibenzo-p-dioxin and related HAHs have previously been reported to affect thyroid function in avian species. Herring gull (Larus argentatus) populations in the lower Great Lakes have displayed evidence of thyroid dysfunction related to HAH exposure. Histopathological examination of thyroid glands from adult herring gulls revealed epithelial hyperplasia, increased mass, and decreases in follicular diameter, epithelial area, and colloid vacuolization when compared to relatively uncontaminated colonies [23]. Spear and Moon [24] observed decreases in serum T3 and T4 concentrations in adult ring doves (Streptopelia risoria) injected i.p. with 3,3',4,4'-tetrachlorobiphenyl, a TCDD-like polychlorinated biphenyl (PCB) congener. A significant negative correlation between plasma free T4 levels and PCB concentrations in yolk was reported in great cormorant (Phalacrocorax carbo) hatchlings from the Netherlands [25]. A positive correlation was observed between plasma total T4 levels and EROD activity in common terns (Sterna hirundo) [26].

Alterations of thyroid hormone levels by TCDD and related chemicals may be due to a number of possible mechanisms. Induction of uridine diphosphate- (UDP-) glucuronosyltransferase by TCDD, known to be under direct regulation of the Ah receptor [21], could cause increased excretion of T4 as the glucuronide conjugate [27]. 2,3,7,8-Tetrachlorodibenzo-p-di-

Table 2. Plasma 17β-estradiol and testosterone concentrations, and estrogen receptor (ER) affinities (Kd) and concentrations (Bmax) in female and male adult great blue herons after 14 d of exposure to a single intraperitoneal injection of 20 µg/kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or corn oil vehicle (mean ± SE).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>n</th>
<th>Control n</th>
<th>20 µg/kg TCDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma testosterone (pg/ml)</td>
<td>Female</td>
<td>2</td>
<td>9.8 ± 0.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5</td>
<td>13.8 ± 4.3</td>
<td>4</td>
</tr>
<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>Female</td>
<td>2</td>
<td>37.8 ± 12.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5</td>
<td>43.8 ± 3.1</td>
<td>4</td>
</tr>
<tr>
<td>Hepatic ER affinity (nM)</td>
<td>Female</td>
<td>2</td>
<td>0.15 ± 0.04</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4</td>
<td>0.15 ± 0.02</td>
<td>4</td>
</tr>
<tr>
<td>Hepatic ER concentration (fmol/mg protein)</td>
<td>Female</td>
<td>2</td>
<td>38 ± 5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4</td>
<td>38 ± 5</td>
<td>4</td>
</tr>
</tbody>
</table>
oxin and related chemicals, and their metabolites, may also compete with T₄ for binding to transthyretin, resulting in the increased clearance and excretion of T₄ [28]. 2,3,7,8-Tetrachlorodibenzo-p-dioxin also possibly influences thyroid hormone levels by altering the activity of type I and/or type II T₄ 5'-deiodinases responsible for conversion of T₄ to T₃ in liver and brain, respectively [22].

Effects of TCDD exposure on plasma sex steroid hormone concentrations and estrogen receptor levels in female and male herons are shown in Table 2. Plasma sex steroid concentrations and hepatic ER levels in female herons were not tested statistically due to the small sample size. Plasma estradiol concentrations were similar among female and male herons. There was no effect of TCDD treatment on plasma estradiol levels in male herons, or in males and females combined. Hepatic ER affinities and concentrations were similar between female and male herons. In addition, hepatic ER concentrations and affinities in adult herons were similar in magnitude to those reported from eggs collected from colonies varying in HAH contamination [14]. However, when separating the adult herons into two groups for this experiment, an approximately equal number of birds from each original location were included in the control and TCDD-treated groups. It is not known what effects, if any, these differences in in ovo HAH exposure had on the herons, especially with respect to failed attempts at breeding, although no obvious differences were observed between birds from the various locations.

In conclusion, although hepatic EROD activity was induced approximately sixfold above control activities in TCDD-exposed adult herons, no consistent effects of TCDD were found on plasma thyroid or sex steroid hormone concentrations, or on hepatic estrogen receptor levels. These results are similar to our previous studies in avian hatchlings exposed in ovo to TCDD, in which these hormonal responses were found to be less sensitive than EROD induction, body weight loss, and embryo lethality [7,8].

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Hormonal effects of acute TCDD exposure in great blue herons


