# *In Vitro* Induction of Ethoxyresorufin O-Deethylase and Porphyrins by Halogenated Aromatic Hydrocarbons in Avian Primary Hepatocytes

**J. Thomas Sanderson[1], Sean W. Kennedy[2] and John P. Giesy[1]**
[1] Department of Fisheries and Wildlife, Pesticide Research Center and Institute for Environmental Toxicology, Michigan State University, 13 Natural Resources Building, East Lansing MI 48824, USA.
[2] National Wildlife Research Centre, Canadian Wildlife Service, Environment Canada, 100 Gamelin Blvd, Hull, Quebec, K1A 0H3, Canada.

## Introduction

Halogenated aromatic hydrocarbons (HAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDD), dibenzofurans (PCDF) and biphenyls (PCB) are highly toxic environmental contaminants. Elevated concentrations of these chemicals in fish-eating birds in the Great Lakes[1] and elsewhere[2-5] have been associated with numerous adverse effects, including reproductive toxicities, embrolethality, deformities, subcutaneous edema and enzyme induction. Laboratory studies have shown that these HAHs have a common mechanism of action which involves initial binding to the aryl hydrocarbon (Ah) receptor[6-8]. A sensitive response that is directly mediated by the Ah receptor is the induction of hepatic cytochrome P-450 1A1 (CYP1A1) and its associated ethoxyresorufin O-deethylase (EROD) activity[9]. Ah receptor binding affinities of HAHs correlate well with their EROD induction potencies *in vitro* and, dependent on the endpoint measured, their toxic potency *in vivo*[7]. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), for example, has a high affinity for the Ah receptor, is a potent inducer of CYP1A1 in a number of cell systems and *in vivo*, and is highly toxic. Therefore, the ability of a chemical to induce CYP1A1 and EROD activity in an *in vitro* system is considered to be a reasonable measure of its toxic potential.

Investigators have examined the EROD induction potential of HAHs in primary hepatocytes of a number of avian species[10-12]. Marked species differences were observed in the sensitivity and magnitude of their response to HAHs, with domestic chicken embryos consistently the most sensitive. These observations are consistent with the species differences among birds in toxic potency of HAHs observed *in vivo*. Chicken embryo hepatocytes were further observed to accumulate high levels of porphyrins upon exposure to TCDD and other HAHs[13,14]. In order to further characterize the sensitivity of these responses to HAHs in avian species, we determined EROD and porphyrin induction potencies of a number of HAHs in primary hepatocytes of hatchlings of the domestic chicken (*Gallus gallus*), herring gull (*Larus argentatus*), ring-billed gull (*L. delawarensis*) and double-crested cormorant (*Phalacrocorax auritus*). The objectives were to: 1) Examine species differences in sensitivity to these responses. 2) Determine inter-individual variabilities in sensitivity. 3) Determine structure activity relationships for several HAHs in each of the species.

## Methods

Fertile eggs of the wild birds species were collected from several locations in the Great Lakes region and incubated artificially in the lab. Birds were sacrificed at hatch. Hepatocytes were prepared from individual livers and cultured in 48-well plates in serum-free medium[14-15]. After 24 h cells were

dosed with HAHs in 2.5 µl isooctane and exposed for 24 h. Then, EROD activity, and total protein and porphyrin contents were measured using fluorescence methods[16]. Each individual hepatocyte preparation was used to obtain a full dose-response curve for TCDD. If sufficient material was available, dose-response curves for any number of HAHs were run in each preparation as well. $EC_{50}$ values of dose-response curves were determined by Woolf plot analysis or visually.

## Results

***Effect of Vehicle on $EC_{50}$ Values for EROD Induction:*** All HAH stock solutions and dilutions in our laboratory are dissolved in isooctane due to the requirements of most experiments. However, DMSO is commonly used by other researchers as a vehicle to dose cells with HAHs. Therefore, we compared the influence of these two vehicles on the EROD induction potency of two HAHs in chicken hatchling hepatocytes (Table 1). TCDD and PCB-77 were both an order of magnitude less potent when administered to the cells in isooctane instead of dimethyl sulfoxide (DMSO). This factor of 10 to 13 was taken into consideration when comparing our $EC_{50}$ values with those of other investigators.

*Table 1. Effect of DMSO or Isooctane as vehicle on the EC50 (nM) of TCDD and PCB-77 for EROD induction in primary chick hepatocytes. EC50 s (mean ± S.D.) were determined in duplicate.*

| | DMSO | Isooctane |
|---|---|---|
| TCDD | 0.008 ± 0.002 | 0.10 ± 0.01 |
| PCB-77 | 0.26 ± 0.01 | 2.7 ± 0.4 |

***EROD Induction by TCDD in Avian Hepatocytes:*** $EC_{50}$s for EROD induction by TCDD were determined in hepatocyte preparations of individual hatchling livers of the ring-billed gull, herring gull and double-crested cormorant. $EC_{50}$ values were found to be highly variable among individual preparations with an apparently skewed distribution around a mean, as illustrated for the double-crested cormorant (Fig. 1). Median $EC_{50}$ values (nM) of TCDD for the induction of EROD activity in the avian hepatocytes were about 0.1 for the domestic chick (n=4), 10 for the cormorant (n=18), and 20 for the herring gull (n=12) and ring-billed gull (n=12). In all three wild species, two or three hepatocyte preparations were found to be non-responsive to TCDD or any other HAHs.

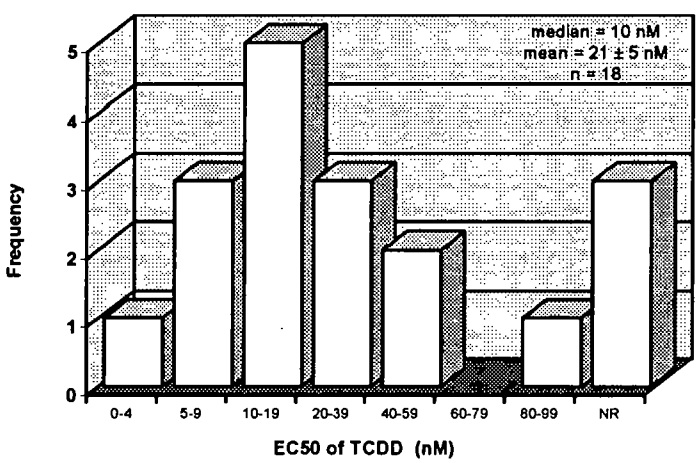


*Fig 1. Distribution of $EC_{50}$ values of TCDD for EROD induction in primary hepatocytes prepared from individual livers of double-crested cormorant hatchlings.*

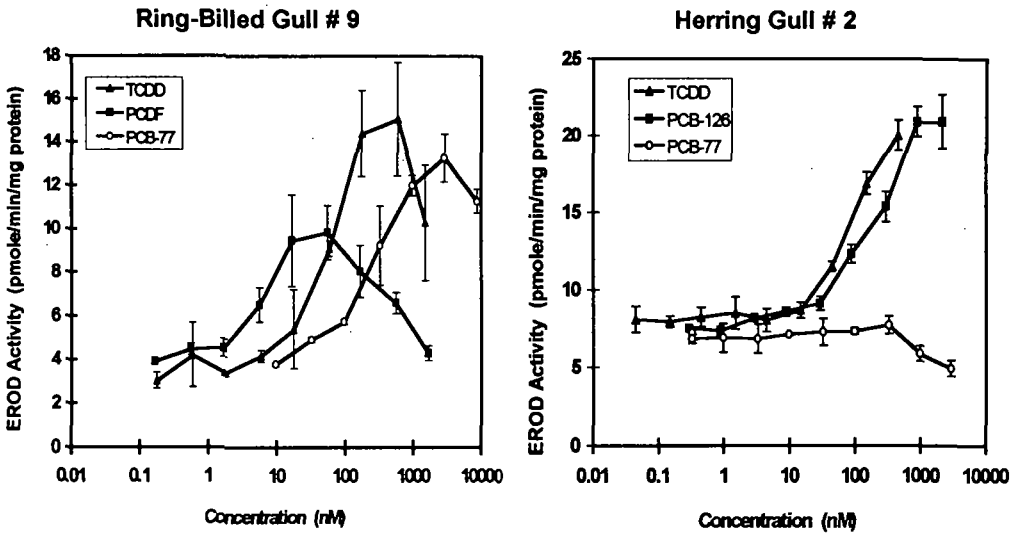


*Fig 2. EROD induction by several HAHs in hepatocyte preparations of an individual liver of the ring-billed gull and herring gull hatchling. Each data point (mean ± S.D.) was determined in quadruplicate.*

***EROD Induction by HAHs in Avian Hepatocytes:*** Typical dose-response curves for EROD induction in a hepatocyte preparation from a single bird liver are shown in Fig 2, for the ring-billed gull (*left*) and herring gull (*right*). The $EC_{50}$ values of these curves were used to determine the potency of each HAH relative to that of TCDD and calculate TCDD-equivalency factors (TEFs) for each of the wild bird species (Table 2).

*Table 2. TCDD-equivalency factors for selected HAHs determined in primary hepatocytes prepared from individual livers of avian hatchlings.*

| HAH | Domestic Chicken[a] | | Herring Gull[a] | | Ring-Billed Gull | Double-Crested Cormorant |
|---|---|---|---|---|---|---|
| TCDD | 1 | | 1 | | 1 | 1 |
| 12378-PCDD | *nd* | | 6.7 - 10 | | 0.5 - 2.7 | 3 - 4 |
| 23478-PCDF | | (1.3) | 1.4 - 4 | | 0.5 - 6.7 | 15 - 20 |
| PCB-126 | 0.3 | (0.2 - 0.3) | 0.2 - 2 | (0.2) | 0.01 - 0.2 | 0.3 - 0.8 |
| PCB-77 | 0.02 | (0.03) | NR - 0.05 | (NR) | NR - 0.2 | NR - 0.1 |
| PCB-81 | *nd* | | 0.8 - 1.2 | | NR - 0.001 | 0.1 - 0.3 |
| PCB-169 | *nd* | (0.02) | *nd* | (0.7) | 0.01 - 0.07 | NR - 0.2 |
| PCB-105 | *nd* | (0.007) | *nd* | | NR - 0.001 | 0.06 |
| PCB-118 | *nd* | (0.0006 - 0.004) | *nd* | (NR) | *nd* | NR |
| HCB | *nd* | | NR | | NR | NR |

NR - non-responsive (no detectable induction within the dose range tested.
*nd* - not determined; HCB - hexachlorobenzene.
a - *TEF values in parentheses were determined in experiments with pooled hepatocytes by refs 11 and 17, in the case of the domestic chicken, and by ref 11, in the case of the herring gull.*

***Porphyrin Induction by HAHs in avian hepatocytes:*** Typically, dose-response curves for the induction of EROD activity in the avian hepatocytes declined in response at high concentrations of the HAH. Generally, the decline in EROD activity coincided with the first measurable increase in porphyrins. This phenomenon was observed with TCDD, 12378-PCDD, 23478-PCDF and PCB-126 in hepatocytes of the domestic chickens and of the wild birds. The magnitude of the porphyrin accumulation response was variable from one hepatocyte preparation to another, from one HAH to another, and from one species to another. The other HAHs were not tested at concentrations sufficiently high to observe a post-maximal decline in EROD activity and increase in porphyrin contents. The determination of lowest observed effect levels for this response are still in progress in our laboratory.

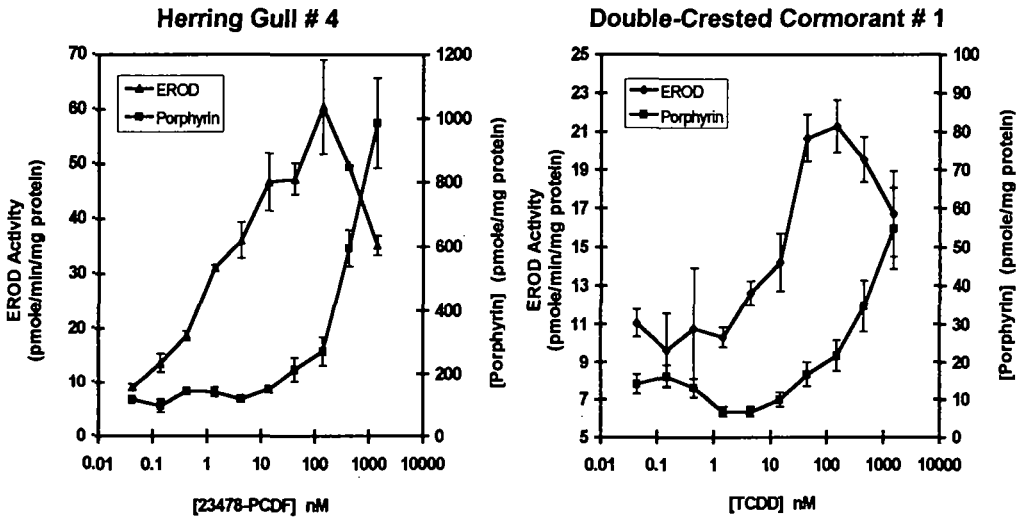


*Fig. 3. Comparison of EROD induction and porphyrin accumulation by PCDF in primary hepatocytes of a herring gull hatchling and by TCDD in a double-crested cormorant hatchling.*

## Discussion

TCDD and other related HAHs induced EROD activity and porphyrins in primary hepatocyte cultures of hatchlings of several avian species. These responses were found to be particularly sensitive to HAH exposure in the domestic chicken, in line with its known great sensitivity to the toxicities of HAHs *in vivo*. Consistent with other reports[10-12], we have found in the present study that these responses are considerably less sensitive to HAHs in some other avian species, such as fish-eating birds. One major difference between our study and those reported previously is that we performed dose-response experiments in hepatocyte preparations of *individual* livers, in order to characterize the inter-individual variability in the sensitivity of these responses; other studies have generally used large pools of livers. A TCDD dose-response curve was generated in every hepatocyte preparation as a positive control, together with any number of HAHs, dependent on the amount of material available. This design was intended to determine the variability in the TEFs for selected HAHs among individuals of each bird species.

Although the experiments shown here are still in progress, we see that individual sensitivity to the prototype inducer TCDD is variable, with $EC_{50}$ values spanning almost 2 orders of magnitude within a given species. Preliminary results from our lab show that pooling two or three livers prior to

hepatocyte preparation considerably reduces the variability in the $EC_{50}$s. Also, $EC_{50}$s determined from TCDD dose-response curves produced in the same hepatocyte preparation varied less than two-fold. This indicates that the variability observed is a reflection of differences in individual sensitivity. In relation to the chicken hatchling, the cormorant is about 100 times less sensitive to *in vitro* EROD induction, and the two gull species about 200 times less sensitive. In another study using primary hepatocytes of hatchlings, the herring gull was found to be about 300 times, and the black-headed gull (*Larus ridibundus*) about 100 times less sensitive than the chicken[11].

Currently, we are extending the study to include a number of other HAHs and another bird species, the Forster's tern (*Sterna forsteri*). We further wish to analyse the yolk sacs of the birds used in the induction experiments for concentrations of PCDDs, PCDFs and PCBs, to see if differences in contaminant burden affect the responses measured *in vitro*. For the purpose of avian risk assessment we also wish to compare the specific TEFs (i.e. structure-activity relationships) for the tested HAHs derived for each bird species to those determined in other bioassays, such as chicken embryo hepatocytes or H4IIE rat hepatoma cells.

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