Effects of In Ovo TCDD Exposure on Wild Avian Species: Summary of a Case Study

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1. INTRODUCTION

The Canadian Wildlife Service (C.W.S.) has monitored great blue heron colonies in British Columbia for many years. Herons provide a good indication of the health of the local marine and estuarine environment because these large, fish-eating birds are near the top of the food chain, over winter in the local area, are colonial, and have been well-studied. In 1987, a colony feeding near the outfall of a kraft pulp and paper mill failed[1]. Out of 57 active nests, no fledglings survived. Chemical analysis of eggs from this colony was carried out as part of the routine monitoring: 3-fold increase in TCDD over the previous year was found. This was superimposed on a steady increase in TCDD for several years beforehand. Our group was formed to study this area along with several other colonies of low and intermediate contamination. We set out to determine which compounds of the many potentially toxic chemicals available might be affecting the environmental health of the birds; to determine what, if any, toxicities could be observed; to study the potential mechanisms involved; to carry out dose-response studies and to develop useful biomarkers of exposure to the toxic chemicals. Our ultimate goal was to calculate the no observed effects level (NOEL) and the lowest observed adverse effects level (LOAEL) in order to provide data for regulatory agencies to use. We also planned to expand the study to include other avian species.

We concluded from our initial studies[2,3] that the major difference between the failed heron colony and the others was the level of TCDD in the eggs and hatchlings. No differences were found in levels of a wide range of compounds including polychlorinated biphenyls, furans, DDE, DDT, etc. Toxic end points correlating with TCDD levels included subcutaneous edema, decreased skeletal growth, decreased down follicles and decreased plasma calcium-concentration. A positive correlation between liver ethoxyresorufin-O-deethylase (EROD) activity, a measure of the cytochrome P-450 1A family, and TCDD was found. **From this data, we developed the working hypothesis that elevated TCDD levels were adversely affecting the reproductive capacity of the heron colony.**

2. OBJECTIVES AND RESULTS

(i) To determine quantitatively various toxicological endpoints for TCDD in the great blue heron and other avian embryos:

The dose-response curves for hepatic EROD activity were completed for great blue herons, double-crested cormorants, chickens and pigeons for TCDD injections in ovo administered (into the air space) during the last third of incubation[4]. From this data we concluded that:

- 1000 ng TCDD/g liver (100 µg/kg egg) produces maximum induction of hepatic EROD in the heron and cormorant chicks with some birds experiencing enough toxicity to inhibit their inducing ability at this dose;
- the ED_{50} for EROD induction is 3-10 µg/kg egg for TCDD in both heron and cormorant eggs. This is 30 to 100 x greater than that required in the domestic chick;
- the no observed effect level of TCDD for EROD induction is 1 µg/kg egg in both wild avian species; the lowest observed effect level is 3 µg/kg egg;
- the pigeon closely resembles the wild avian species re EROD induction;
- the lowest observed effect level for "chick edema" (a classic TCDD toxicity in birds) is 3 µg/kg for the cormorant; that is, it occurs at the same level as EROD induction. However, in heron chicks, the lowest observed effect level for edema was 0.5 µg/kg, below the LOED for EROD induction (3 µg/kg).

This provides further evidence that EROD induction, as a measure of the activation of the Ah receptor, is a convenient marker for potential Ah-receptor mediated toxicities. Dependent on the genetic make-up of the species, some toxicities may have already begun.
It should be noted that in all of the above experiments the TCDD injections were given 5 to 7 days before hatching because of the exquisite sensitivity of the embryos to the lethal effects of this chemical, and the limited availability of wild avian eggs to conduct large-scale experiments. We developed the pigeon as a good control species for the heron and cormorants: as a result, we were able to begin studies where the TCDD was administered at earlier time points during incubation. This provides a more realistic model to that which occurs in the environment. Using our control species, TCDD was injected into pigeon eggs on embryonic day 4 (E4) to study the effects of earlier exposure of the embryo to the chemical. The time periods studied were at hatch and day 7 after hatching; a series of doses of TCDD were used. A number of potentially

less invasive endpoints were monitored: plasma triiodothyronine, thyroxine, estradiol and testosterone, along with the usual endpoints. EROD was induced 15- and 6-fold on hatch and day 7, respectively at a dose of 1 µg/kg. There were significant decreases in yolk-free body weight and skeletal growth in these chicks. However no significant or consistent differences were noted in plasma hormone levels of the treated groups as compared to their controls. Higher doses resulted in significant lethality (see below). When the experiment was repeated using great blue heron eggs and a dose of 2 µg/kg TCDD at E13, hepatic EROD was induced 3- and 2-fold at hatch and day 7. Decreased skeletal growth was found, but no significant or consistent changes in plasma hormone levels occurred.
CONCLUSIONS:

- **significant toxic endpoints occur - e.g. decreased skeletal growth**
- **hepatic EROD is a sensitive biomarker**
- **plasma thyroid and sex hormones cannot be used as biomarkers**
- **slight increases in TCDD dose during early time periods result in large increases in embryonic mortality.**

(ii) To determine the lethality of TCDD in great blue heron and other avian embryos:

Embryos are highly sensitive to TCDD[4]. In the pigeon, a 50% increase of mortality over control levels occurred at a TCDD dose of 3.0 µg/kg administered 5 to 7 days before hatching. At 100. µg/kg, the mortality more than doubled again. The LD_{50} was estimated to be approximately 100. µg/kg in the pigeon; the LD_{20} was approximately 10 µg/kg. Significant mortality (LD_{20}) occurred in the great blue heron embryos at 1.0 µg/kg and in cormorants at 3.0

µg/kg (TCDD administered at 5 to 7 days before hatching). As expected, the domestic chicken embryo was the most sensitive species, with an LD_{20} of approximately 0.2 µg/kg.

However, when the TCDD was administered on day 3.5 of incubation (the earliest time point at which we can establish fertility of the egg), the LD_{20} in pigeons was approximately 1. µg/kg; the LD_{50} was 2.4 µg/kg. That is, at this earlier time period (dosed at 3.5 days incubation) the embryo was between 1 and 2 orders of magnitude more sensitive to the lethal effects of TCDD than at the later time period (dosed at 5-7 days before hatching). These data are particularly important in the interpretation of our dose-response curves for the embryos exposed to environmental contamination (for the entire embryonic period) vs the laboratory controlled studies (TCDD for the last 5-7 days of development only).

CONCLUSION: **Decreased hatchability would be expected to occur at TCDD levels of 0.3 µg/kg, which was the level found in the contaminated environments exhibiting decreased reproductive success.**

(iii) ***To measure TCDD distribution in avian embryos:***

In a ^{3}H-TCDD distribution study, we found that there were no differences in TCDD concentration among the 4 species studied[4]. In particular, the liver contained 6-8% of the total dose in all 4 species, proving that a distributional difference did not explain the different sensitivities noted. We also followed the distribution of ^{3}H-TCDD injected into the egg yolk vs that injected into the air sac. Again, no distributional differences were found, so that further studies have all been done via the air sac, for convenience and accuracy.

(iv) ***To determine if there are measurable effects related to TCDD toxicity in heron and cormorant embryos and/or chicks hatched under laboratory conditions where eggs are from colonies exposed to high and low environmental pollution:***

Cormorant eggs were collected from five colonies across Canada with differing levels of contamination[5]. Levels of contamination expressed in total sum of TCDD-toxic equivalents (TEQ) (ng/kg egg; mean ± SEM) were: Saskatchewan (250 ± 50), Chain Islands (672 ± 73), Christy Islet (276 ± 14), Crofton (131; n=1) and Lake Ontario (1606 ± 118). In the hatchlings, hepatic EROD activities (pmole/min/mg protein; mean ± SEM) were: Saskatchewan (283 ± 42), Chain Islands (516 ± 98), Christy Islet (564 ± 91), Crofton (391 ± 52) and Lake Ontario (2250 ± 156). Hepatic microsomal EROD activity regressed positively on TEQ; yolk weight regressed negatively on TEQ. Wing length regressed negatively on PCB-169. **Monospecific antibodies raised against rat cytochrome P-450 1A1 recognized a protein in the hepatic microsomes of the cormorant, and also of the heron using immunoblotting.** The intensity of the stained band increased with increased EROD activity thus supporting the assumption that ethoxyresorufin is a suitable substrate for avian cytochrome P-450 1A1.

CONCLUSION: **These results validate the use of avian hepatic microsomal EROD activity as an index of cytochrome P-450 1A1 induction by environmental levels of polychlorinated aromatic hydrocarbons and as a useful screening tool to determine the extent of exposure to such chemicals. Furthermore, the induction of cytochrome P-450 1A1 observed in the cormorant indicates that the Ah receptor-mediated process, by which TCDD and related chemicals exert many of their toxicities, has been activated.**

In another study, we **examined the applicability of EROD activity as a temporal bioindicator of chemical contamination of the habitat of an avian species[6].** The effect of chemical contamination with TCDD and other similarly acting chemicals was examined on

hepatic microsomal EROD activities in the chicks of great blue heron eggs collected from two colonies in B.C. between 1990 and 1992. Several morphological parameters were measured in order to examine effects on growth and development. We compared these results with those found in great blue heron chicks from the same colonies studied in 1988. **The aim was to confirm our previous findings and to determine whether the effects observed in this study behaved according to the dose-effect relationships determined in 1988, particularly since the process changes implemented by the Crofton pulp and paper mill between 1988 and 1990 led to decreased discharge of PCDDs and PCDFs into the Strait of Georgia**. Heron eggs were collected from the Vancouver colony in 1990 and 1992, and from Crofton in 1991. After hatching, biological parameters in the hatchlings and chemical contaminant levels in the paired eggs were measured and compared with the findings from the same colonies studied in 1988. **The levels of TCDD and other PCDDs and PCDFs had decreased significantly in both colonies since 1988. A concomitant decrease in EROD activity, decrease in incidence of chick edema, and improvement of reproductive success of the Crofton colony was observed.** TCDD level was significantly correlated with body, yolk-free body, kidney, intestine, stomach and tibia weights; tibia length; and hepatic EROD activity, confirming the findings of 1988. Correlating the sum of TCDD-toxic equivalents (TEQs) with these parameters resulted in similar correlations. Of particular interest was the finding that the correlations were maintained when only one colony (Vancouver) was analyzed over the 5 year time period. This rules out error due to genetic variability.
CONCLUSION: **Symptoms known to be associated with TCDD parallel the increases and decreases of TCDD found in the environmentally contaminated eggs.**

(v) To determine the kinetics of TCDD binding to avian Ah receptors:

The induction of hepatic EROD occurs mechanistically through the Ah receptor. In order to further delineate the mechanism responsible for the varying sensitivities of the 4 avian species to TCDD and similar chemicals, the Ah receptor binding capacity and affinity was measured in hepatic cytosol[4]. We found specific binding of TCDD in the 9-10 S region of sucrose density gradients, comparable to that of human cytosolic Ah receptor protein. The binding affinity of the domestic chick Ah receptor was 10 x greater than that in the pigeon, heron or cormorant chick. Therefore, this accounts for much of the approximately 30-fold difference in sensitivity to TCDD which we saw in the dose-response curves. Of interest re risk assessment calculations, is the fact that the Ah receptor ligand binding affinity of human placenta is between that of the domestic chick and the wild avian species. **That is, the receptor in human placenta has an approximately two-fold higher affinity than that of the wild "sentinel" birds.**
CONCLUSION: Avian embryoes exhibit an Ah receptor with similar binding affinity to human placenta.

3. **In summary, the major achievements of our research on heron and cormorant eggs exposed to TCDD in ovo are:**

- validation of the use of the hepatic EROD assay in two wild avian species as a bioindicator of contamination by relatively high environmental levels of 2,3,7,8-TCDD.
- corroboration of EROD as a bioindicator following decreased environmental contamination due to pulp mill process changes.
- evidence that avian EROD activity is due to cytochrome P-450 1A1.

- evidence that Ah receptor affinity accounts for most of the difference in relative sensitivities of the various avian species to the biological effects of TCDD.
- a rank ordering of Ah receptor affinity (chick liver > adult rat liver > human placenta > pigeon ≈ heron ≈ cormorant chick liver).
- TCDD resulted in no change in plasma thyroid or sex hormone levels; that is, these plasma hormone analyses could not be used as a non-invasive biomarker.
- exposing the avian embryos to TCDD during the last quarter of incubation, we determined dose-response curves for several endpoints, and estimated the NOEL, LOAEL and ED_{50}.
- exposing the avian embryos to TCDD starting from embryonic day 4 resulted in a 10 fold increased sensitivity to lethality, with extremely acute dose-response curves. (LD_{20} and LD_{50} were estimated).

We conclude that in the Georgia Straight area of B.C., 2,3,7,8-TCDD had built up to environmental levels which were at the lower end of the linear steeply rising portion of the dose-response curves in several wild avian species, such that toxicities were starting to become evident. When remedial process changes decreased the chemical input into the environment, fairly rapid positive responses were noted in the avian population. Therefore, herons and cormorants are reasonable biomonitors of environmental health. In addition, measures taken to protect the health of these species are likely to impinge positively on human health.

It should be stressed that our results are from an environmental case study where the major polychlorinated hydrocarbon was TCDD and where this chemical was present in relatively high levels. Clearly, each area and each pulp mill, may provide a different set of conditions.

4. REFERENCES

1) Elliott, J.E., R.W. Butler, R.J. Norstrom and P.E. Whitehead (1989): Environmental Contaminants and Reproductive Success of Great Blue Herons Ardea herodias in British Columbia, 1986-87. Environ. Pollut. 59, 91-114.

2) Bellward, G.D., R.J. Norstrom, P.E. Whitehead, J.E. Elliott, S.M. Bandiera, C. Dworschak, T. Chang, S. Forbes, B. Cadario, L.E. Hart and K.M. Cheng (1990): Comparison of Polychlorinated Dibenzodioxin Levels with Mixed Function Oxidase Induction in Great Blue Herons. J. Toxicol. Envir. Health. 30, 33-52.

3) Hart, L.E., K.M. Cheng, P.E. Whitehead, R.M. Shah, R.J. Lewis, S.R. Ruschkowski, R.W. Blair, D.C. Bennett, S.M. Bandiera and G.D. Bellward (1991): Effects of Dioxin Contamination on the Growth and Development of Great Blue Herons. J. Toxicol. Envir. Health 32, 331-344.

4) Sanderson, J.T. and G.D. Bellward (1994): Hepatic Microsomal Ethoxyresorufin O-deethylase-Inducing Potency in ovo and Cytosolic Ah Receptor Binding Affinity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin: Comparison of Four Avian Species. Toxicol. Appl. Pharmacol. (in press).

5) Sanderson, J.T., R.J. Norstrom, J.E. Elliott, L.E. Hart, K.M. Cheng and G.D. Bellward (1994): Biological Effects of Polychlorinated Dibenzo-p-dioxins, Dibenzofurans and Biphenyls in Double-Crested Cormorant Chicks (Phalacrocorax auritus). J. Toxicol. Envir. Health, 41, 247-265.

6) Sanderson, J.T., J.E. Elliott, R.J. Norstrom, P.E. Whitehead, L.E. Hart, K.M. Cheng and G.D. Bellward (1994): Monitoring Biological Effects of Polychlorinated Dibenzo-p-dioxins, Dibenzofurans and Biphenyls in Great Blue Herons in British Columbia. J. Toxicol. Environ. Health, 41, 435-450.