Morphometric Abnormalities in Brains of Great Blue Heron Hatchlings Exposed in the Wild to PCDDs

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Great blue heron hatchlings from colonies in the Strait of Georgia, British Columbia, Canada are being monitored for environmental contaminant exposure and effects by the Canadian Wildlife Service. The contaminants of concern are polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), primarily derived from kraft pulp mill effluent. The levels of PCDDs and PCDFs in eggs from the most contaminated colonies peaked in 1988 and 1989 and dropped dramatically through 1990 to 1992. Brains of heron hatchlings (taken as eggs from the wild and hatched in the laboratory) were analyzed for gross morphological abnormalities. Brains from highly contaminated colonies (Crofton, British Columbia and University of British Columbia Endowment Lands) in 1988 exhibited a high frequency of intercerebral asymmetry. The frequency of this abnormality decreased in subsequent years as the levels of TCDD and TCDD-TEQs (toxic equivalence factors) decreased. The asymmetry was significantly correlated with the level of TCDD and TCDD-TEQs in eggs taken from the same nest. Yolk-free body weight negatively correlated and the brain somatic index positively correlated with the TCDD level in such pair-matched eggs. These results indicate that gross brain morphology, and specifically intercerebral asymmetry, may be useful as a biomarker for the developmental neurotoxic effects of PCDDs and related chemicals. — Environ Health Perspect 103(Suppl 4):61–66 (1995)

Key words: polychlorinated dibenzodioxin, polychlorinated dibenzofuran, development, birds, asymmetry, TCDD, morphology, central nervous system, brain, kraft pulp mill effluent

Introduction

The province of British Columbia in Canada has a resource-based economy. Forests are one of the main resources. Thus, the forestry and pulp and paper industries are the largest industrial base for the province. Most of the pulp and paper companies of British Columbia use the bleached kraft process, which ultimately releases polychlorinated dibenzodioxins (PCDDs; including 2,3,7,8-tetrachlorodibenzodioxin [TCDD], 1,2,3,7,8-pentachlorodibenzodioxin, and 1,2,3,6,7,8-hexachlorodibenzodioxin) and polychlorinated dibenzofurans (PCDFs; including 2,3,7,8-tetrachlorodibenzofuran) in the effluent. As a result of the persistent halogenated aromatic hydrocarbon (HAH)-related industrial contaminants in the Strait of Georgia (off the west coast of the mainland of British Columbia), the most significant are the PCDDs and PCDFs, with only a minor contribution of polychlorinated biphenyl (PCB) from other industries. Analysis of great blue heron (*Ardea herodias*) eggs from a colony near a pulp mill in British Columbia in 1988 showed that PCBs contributed less than half of the total TCDD-like activity as measured by toxic equivalents [TCDD-TEQs; (*1,2*)]. This situation contrasts strongly with that in the Great Lakes, where PCBs contribute most of the TCDD-like activity in piscivorous birds such as the double-crested cormorant (*Phalocrocorax auritus*) (*3*). The Strait of Georgia, British Columbia is thus an ideal location to study the effects of PCDDs and PCDFs on wildlife.

Since 1982, the Canadian Wildlife Service (CWS) has been monitoring the levels of PCDDs and PCDFs in the Strait of Georgia, in part by evaluating the levels of these compounds in the eggs of great blue herons. Since 1983 they have also been monitoring the reproductive success of the heron colonies (*4*). In 1987 and 1988 one colony failed to produce any fledglings. This colony is located near a pulp and paper mill in Crofton on Vancouver Island. The Crofton colony also had the highest levels of PCDDs in the eggs. Between 1988 and 1990, the Crofton pulp mill instituted major changes in their pulp processing that has led to a decrease in the level of PCDDs and PCDFs in the effluent released into the Strait of Georgia (*5*).

Evaluations of the various heron colonies and of hatchlings taken (as eggs) from the colonies in 1988 indicated that *a*) there were significant colony differences in yolk-free body weight, kidney weight, stomach weight, tibia dry and ash weight,

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tibia and beak length, and down follicle density (as examined on the head) (*6*); *b*) all of the above differences also correlated negatively with log TCDD concentrations in eggs taken from the same nests as the evaluated hatchlings; *c*) analysis of P450 enzyme activity indicated that ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-deethylase (PROD) activity also varied by colony, being highly induced in the more contaminated colonies (*7*); and *d*) the behavior of the parent herons in the most contaminated colony (Crofton) differed from the behavior of the herons in the control colony; nesting was less synchronized from nest to nest, and nest attentiveness was lower and more variable at the Crofton colony compared to Sydney Island (a control colony) (*8*).

Since the nervous system interacts with the endocrine system to control reproduction, growth, and behavior, we initiated studies to evaluate whether the brain was affected in the herons from the more contaminated colonies. Preliminary observations (*9,10*) on brains from heron hatchlings taken in 1988 and 1989 (as eggs) from colonies with low, moderate, and high levels of PCDD and PCDF contamination indicated that *a*) many of the hatchling brains developed a gross cerebral asymmetry that varied in frequency by colony and that increased in frequency and degree with increasing levels of TCDD in eggs taken from the same nests as the evaluated hatchlings; *b*) there was a significant increase in the cell density and width of the pyriform cortex of the forebrains of heron hatchlings taken from the most contaminated colony (Crofton) compared to a relatively uncontaminated "control" colony. Many of the cells that were increased in number were immunoreactive for taurine, a putative inhibitory neurotransmitter); and *c*) brain weight decreased with increasing levels of TCDD. At this time it is unclear what relationship these changes have to the growth, behavioral, and reproductive changes seen in the contaminated heron hatchlings. No one has fully identified how the pyriform cortex, for example, is controlled or how it is linked into many of the other structures in the avian brain. However, the cerebral hemispheres contain the integrative part of the brain. The pyriform cortex (on the lateral aspects of the cerebral hemispheres) is an accessory structure for the limbic system, which controls, among other things, emotions and instinctive behavior. In addition, the pyriform cortex is the second structure to receive olfactory input, which in many animals plays a role in instinctive behavior (*11*).

The present experiments extend these preliminary observations to the subsequent years when, due to improvements in the kraft process by the local pulp mills, the overall contamination level of PCDDs and PCDFs in the Strait of Georgia decreased to moderately low "background" levels, as indicated by the amount of contamination of heron eggs (*2*).

Materials and Methods

Methods were as described in detail in Henshel et al. (*9*). Briefly, two eggs from each nest were collected by the Canadian Wildlife Service. One egg was taken for analysis (individual or pooled as indicated) and one egg was incubated until hatching in the laboratory. These eggs were termed pair-matched eggs. All birds were euthanized within 24 hr of hatching. The brains were removed and preserved in 100 ml 4% paraformaldehyde (4°C), and subsequently transferred to phosphate-buffered saline containing azide for storage. Brains were weighed postfixation to reduce risk of damage to the brains during the weighing process.

Eggs were taken from a total of five colonies: Nicomekl, University of British Columbia Endowment Lands (UBC), and Crofton in 1988, Chilliwack and UBC in 1990, Nicomekl, UBC, Crofton, Chilliwack, and Tillicum in 1991, and UBC in 1992. The Nicomekl, Chilliwack, and Tillicum colonies were considered to be our reference, i.e., less contaminated, colonies.

Brains were measured by eye, using an engineering ruler (with 0.5 mm gradations) held parallel to the plane of measurement. Measurements that fell between the gradations were recorded as 0.25 mm. All difference measurements used reflect the right brain measurement subtracted from the left brain measurement. Care was taken to have the brains measured and weighed by the same person to reduce inconsistencies between observations. Brains were coded when measured. The measurements were subsequently decoded and analyzed by a different person.

PROC GLM (General Linear Model) and PROC REG (Linear regression) (SAS 6.04; SAS Institute; Cary, North Carolina) were used to analyze the data. Measurements were compared to the source colony (nested in year) and TCDD and TCDD-TEQ values from eggs taken from the same nest. TCDD-TEQs were calculated based on PCDD and PCDF measured values using Safe's toxic equivalence factors (*1*). Significance was determined using an α of 0.05 and is expressed as the probability of occurrence of the null hypothesis. Mean values ± standard error of the mean (SEM) were calculated and are listed in Table 1 along with sample sizes. Some chemical analyses were made on pooled samples. These are indicated in Table 1.

Results

Cerebral Asymmetry

Normal avian brains typically are grossly symmetrical. These brains appear asymmetrical in that there is a greater convexity in the left, compared to the right, cerebral hemisphere. To quantify the observable brain asymmetry (Figure 1), we made four measurements of the forebrain region (Figure 2). The width, height, and angle measurements are only affected by brain nuclei (functional aggregates of neurons) in the cerebral hemispheres. The depth measurement, however, is also affected by the rostral thalamic and hypothalamic nuclei, especially the preoptic area (POA).

Of the four measurements made, only the width, angle, and depth measurements varied significantly with colony nested in year ($p < 0.0001$ for all three measurements;

Table 1. SEMs and sample sizes (in parentheses) for each experimental variable used in Figures 3, 5, and 8.

Year	Colony	Dif width, mm	Dif angle, mm	Dif depth, mm	TCDD, ppt	TEQ, ppt	Brain wt, g	BSI
1988	N	0.011 (8)	0.000 (8)	0.000 (8)	0.22 (8)	0.77 (8)	0.004 (8)	0.003 (8)
1988	UBC	0.034 (9)	0.033 (9)	0.029 (9)	4.92 (9)	13.64 (9)	0.012 (9)	0.003 (9)
1988	Cr	0.023 (8)	0.029 (8)	0.044 (8)	8.81 (8)	14.63 (8)	0.005 (8)	0.004 (8)
1990	UBC	0.011 (7)	0.044 (7)	0.017 (7)	4.51 (7)	7.65 (7)	0.010 (7)	0.005 (7)
1990	Ch	0.000 (4)	0.000 (4)	0.000 (4)	0.00 (4) [a]	0.00 (4) [a]	0.02 (4)	(4) [b]
1991	N	0.000 (2)	0.000 (2)	0.000 (2)	(2) [b]	(2) [b]	0.01 (2)	0.000 (2)
1991	UBC	0.024 (4)	0.000 (4)	0.029 (4)	0.00 (4) [a]	0.00 (4) [a]	0.02 (4)	0.006 (4)
1991	Cr	0.005 (14)	0.013 (14)	0.004 (14)	1.09 (14)	3.67 (14)	0.01 (14)	0.002 (14)
1991	T	0.000 (2)	0.000 (2)	0.000 (2)	0.46 (2)	0.00 (2)	0.00 (2)	(1) [b]
1992	UBC	0.000 (8)	0.000 (8)	0.000 (8)	0.46 (8)	0.99 (8)	0.01 (8)	0.003 (8)

[a]Pooled data. [b]Missing data.

$r^2 = 0.60$, 0.69, and 0.41, respectively; Figure 3). For all three measurements, the asymmetry was significantly greater in the brains from herons from the UBC and Crofton colonies in 1988 compared to either the Nicomekl colony in 1988, or any of the colonies in subsequent years ($p < 0.001$–0.0001, depending on the measurement). Figure 4 illustrates the mean TCDD (*A*) and TCDD-TEQ (*B*) values in the pair-matched eggs for each colony by year. Analysis of variance indicated a significant relationship between the TCDD and TEQ values and the colony nested in year ($r^2 = 0.80$, 0.74, respectively; $p < 0.0001$). Note that as the TCDD and TCDD-TEQ levels decreased in the most contaminated colonies (Crofton and UBC), the degree of cerebral asymmetry correspondingly decreased. This relationship is emphasized in the scattergrams plotting TCDD against the width and angle difference measurements and TCDD-TEQ against the depth difference measurement (Figure 5*A,B,C*, respectively). As the TCDD (or TCDD-TEQ) levels increased in eggs taken from the same nest, the likelihood and degree of observed asymmetry also increased. (Log TCDD and log TCDD-TEQ are used for the X axes because, when the measurements are regressed against the dose or log dose alone, the log dose correlation coefficient is higher. The correlation coefficients (r^2) for each of these relationships are: width: 0.57 (TCDD), 0.42 (TCDD-TEQ); angle: 0.61 (TCDD), 0.46 (TCDD-TEQ); and depth: 0.40 (TCDD), 0.44 (TCDD-TEQ).

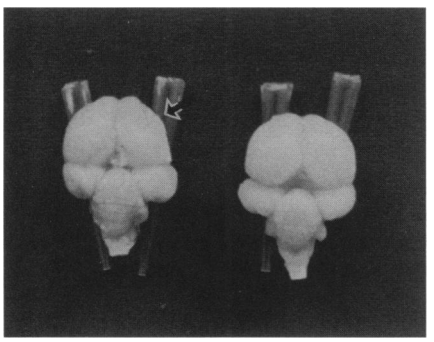

Figure 1. Photograph of the dorsal aspect of brains taken from the UBC colony (left) and the Chilliwack colony (right) in 1990. The arrow points to the right hemisphere of the brain of a heron from the UBC colony; it has a flatter arc compared to the right hemisphere of the brain from a heron from the Chilliwack colony. The three-dimensional increased convexity in the left hemisphere does not visualize well from this angle.

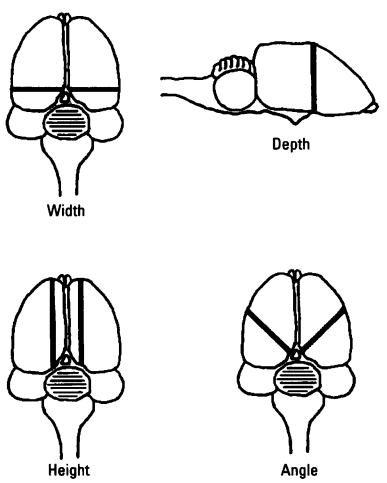

Figure 2. Diagram of the four measurements made. The width, height, and angle measurements (drawn in dorsal view) were all made with the brain lying parallel to the desktop. The depth measurement (drawn from the lateral view) was made with the brain lying coronally perpendicular to the desktop.

The regression equations with the highest r^2 value that best describes the interactions of the factors believed to be affecting the brain asymmetries are

$$\text{width difference} = 0.002 \times \text{TCDD} + 13.897 \times \text{brain wt/body wt} - 0.353$$
$r^2 = 0.61$, $p < 0.0001$

$$\text{angle difference} = 0.459 \times \log \text{TCDD} - 0.456$$
$r^2 = 0.69$, $p < 0.0001$;

$$\text{depth difference} = 0.001 \times \text{TCDD} - \text{TEQ} + 13.619 \times \text{brain wt/body wt} + 0.010$$
$r^2 = 0.49$, $p < 0.0001$.

Brain and Body Weights

As seen previously (*9*), the body weight and yolk-free body weight decreased with increasing TCDD concentrations in the pair-matched egg (Figure 6).

The regression equation for this relationship is

$$\text{yolk-free body weight} = -3.853 \times \log \text{TCDD} + 51.797$$
$r^2 = 0.19$, $p < 0.001$.

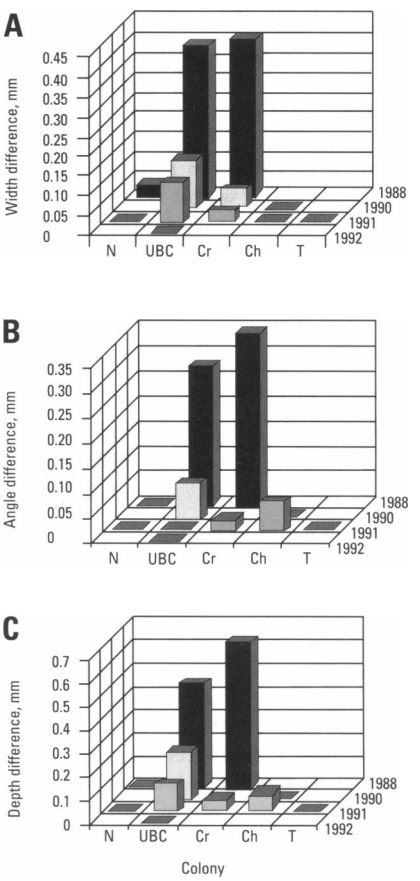

Figure 3. Histograms illustrating the mean values for the differences in width (*A*), angle (*B*) and depth (*C*) of the heron brains analyzed by colony and year. Of the colonies, N is Nicomekl, UBC is the colony near the University of British Columbia, Cr is Crofton, Ch is Chilliwack, T is Tillicum. SEMs and sample size for each measurement are listed in Table 1.

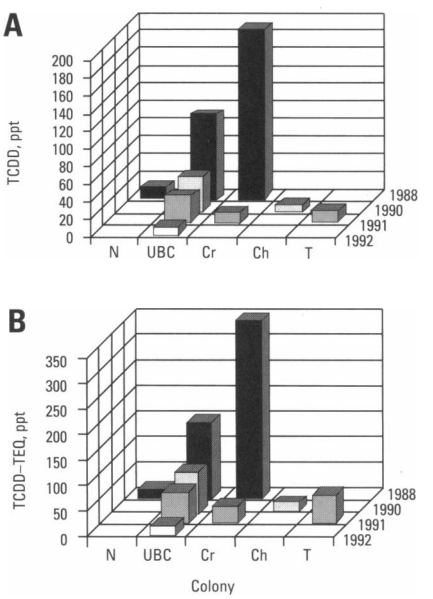

Figure 4. Histograms illustrating the mean TCDD and TCDD-TEQ levels in each colony in each year as determined in an egg taken from the same nest as each egg/hatchling used in these measurements. SEMs and sample sizes are listed in Table 1. SEMs of 0 indicate pooled samples were used for the measurements of those colonies in those years.

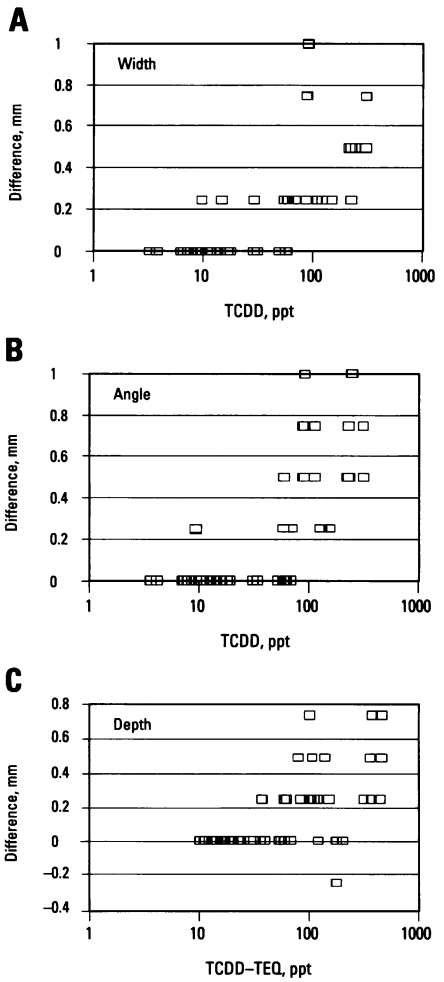

Figure 5. Scattergrams illustrating the relationship between TCDD (*A*, width; *B*, angle) and TCDD-TEQ (*C*, depth).

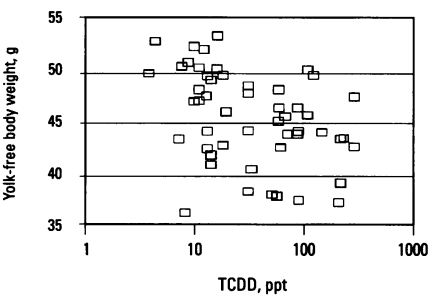

Figure 6. Scattergram depicting the relationship between yolk-free body weight and TCDD levels. The correlation coefficient for this relationship is –0.34.

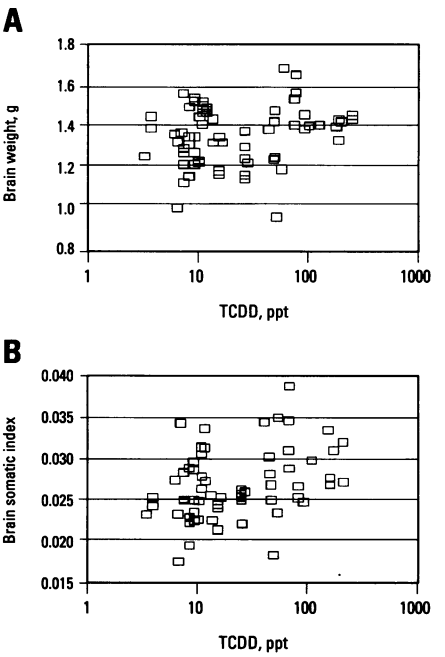

Figure 7. Scattergrams relating brain weight (*A*) and Brain somatic index (BSI, *B*) to log TCDD levels. The correlation coefficients for these relationships are 0.05 (brain weight) and 0.31 (BSI).

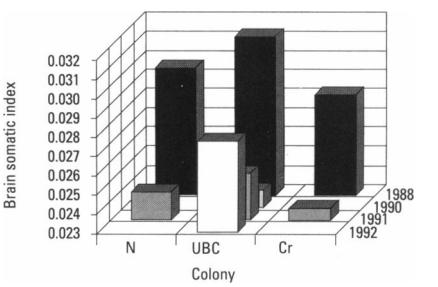

Figure 8. Histogram of the mean brain somatic index in the heron brains averaged by colony and year. SEMs and sample size for each mean are listed in Table 1.

Brain weight and the brain somatic index (BSI, brain weight/body weight), both tend to increase with increasing TCDD and TCDD-TEQ levels (Figure 7*A*,*B*; $p < 0.0001$ for log TCDD vs BSI). This was not clear from the 1988 results in which there was a significant decrease in brain weight in the brains of herons from the Crofton colony (the colony in which there were the highest contamination levels) compared to brains of herons from both the Nicomekl (low contamination) and UBC (moderate contamination) colonies (Figure 8). However, as the TCDD levels decreased in subsequent years, there was a decrease in the brain weight and the brain somatic index. Unlike the clear relationship between the BSI and log TCDD, the relationship between brain weight and log TCDD is not so clear. Log TCDD only varies significantly with brain weight ($p < 0.0001$) when the colony-related factors are also included as covariates in the analysis of (co)variance. One contribution to the change in brain weight may be the change in body weight. In general, when bodies decrease in size, the organs decrease correspondingly. However, the brain weight has a tendency to increase with increasing TCDD, a factor that decreases body weight. The majority of the variation in brain weight is due to the colony-related factors (evaluated as colony nested within year; $r^2 = 0.97$; $p < 0.0001$). When considering the contaminant-related increase in the brain somatic index, the decrease in body weight is also a significant factor ($r^2 = 0.85$, $p < 0.0001$). Since body weight is one of two factors in the BSI, it was not included in the regression equation to avoid a problem with collinearity.

The regression equation relating BSI to log TCDD and colony nested within year ("colony by year") is

$$\text{Brain somatic index} = 0.003248 \times \log \text{TCDD} - 0.0000011 \times (\text{Colony by year}) + 0.02754$$

$r^2 = 0.49$ $p > 0.0001$

Discussion

We have shown that heron embryos exposed *in ovo* to a mixture of PCDDs and PCDFs hatch with asymmetric brains. This effect has appeared not only in comparison of colonies with different degrees of exposure in the same year, but also in the same colony as the level of contamination decreased over time. This phenomenon, which is easily quantifiable, could be used as a morphometric biomarker for TCDD-related exposure.

The brain asymmetry also provides an indication of how the PCDDs and PCDFs interact with the brain. The width and angle measurements, which are only affected by the cerebral hemispheres, correlate best with the TCDD (or log TCDD) levels, while the depth measurement (which is affected by the steroid-sensitive anterior hypothalamus) correlates best with the TCDD-TEQ levels. Thus, it is possible that different PCDD and PCDF congeners have differing effects or different potencies in the individual brain regions potentially related to the steroid sensitivity of the region.

In addition to the TCDD-related brain asymmetry, the BSI tended to increase with increasing TCDD concentrations in the pair-matched egg. The corresponding decrease in body weight probably affected this relationship. The BSI also tended to vary among colonies, indicating that some other factor besides simply TCDD levels might be influencing brain weight. These

factors might include genetic differences, gender (which was not determined, as the hatchling gonads are undifferentiated), hormonal status, nutrition, or other unmeasured contaminants.

As expected, the yolk-free body weight also decreased with increasing TCDD and TEQ values. Hoffman et al. (*12*) first suggested that yolk-free body weight could be used as a biomarker for the effects of PCBs on the growth of heron embryos. They noted that yolk-free body weight decreases with increasing PCB concentration, as noted in this dataset as well.

TCDD is arguably the most toxicologically potent of the group of related halogenated aromatic hydrocarbons (HAHs), which includes PCDDs, PCDFs, and PCBs. Most biomarkers of toxicity that are now in use to assess the effects of TCDD (and related chemicals) on developmental processes evaluate teratogenic, cellular, and/or biochemical changes in the palate and beak, kidney, liver, spleen, bursa, thymus, thyroid, gonads, white blood cells, and blood hormone and vitamin levels (estrogen, androgens, thyroid hormone [T_3,T_4], and vitamin A) (*13–17*). Other previously noted effects include pericardial and abdominal edema and (as mentioned above) decreased body weight and growth (*18,19*). Several studies have noted that exposure to TCDD-related chemicals is correlated with changes in behavior, especially reproductive and parenting behaviors (*16,20*). As behavior is largely controlled by the central nervous system, it is important to find and develop biomarkers for central nervous system toxicity that may represent the anatomical correlates of the behavioral changes. Such a nervous system biomarker will both validate the behavioral changes and potentially provide an indication for where in the nervous system the chemical is acting.

Recent studies by Seegal et al. (*21*) have indicated that HAH-correlated changes in nigro-striatal dopamine levels are most potently induced by the -*ortho*-substituted, noncoplanar PCBs (2,2′-dichlorophenyl and 2,2′,4-trichlorophenyl). These PCBs are toxicologically dissimilar to the meta and para (laterally)-substituted, coplanar analogs of TCDD when evaluated using a number of different assays (*1*). In this study, the forebrain asymmetry (width and angle measurements) correlates best with the TCDD levels in the pair-matched egg. Yet the variation in the depth measurement and its higher correlation with the TCDD-TEQ values may reflect the contribution of other, nonlaterally substituted PCDDs and PCDFs. It is important to recognize, however, that even though a number of PCDDs and PCDFs are contributing to the determined TEQs (*1*), all PCDDs and PCDFs are inherently coplanar. The response of the central nervous system to the PCDDs, PCDFs, and PCBs may include more than one response potentially mediated by a variety of the coplanar and noncoplanar halogen-substituted congeners. It becomes therefore even more important to understand the multiplicity of mechanisms by which the HAHs interact with the central nervous system.

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