A Mixture of Dioxins, Furans, and Non-ortho PCBs Based upon Consensus Toxic Equivalency Factors Produces Dioxin-Like Reproductive Effects

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD; dioxin) and related polyhalogenated aromatic hydrocarbons (PHAHs) alter the reproductive development of laboratory animals. Therefore, we exposed animals to a mixture of dioxins, furans, and polychlorinated biphenyls (PCBs) that included TCDD, 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PCDD), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (1-PeCDF), 2,3,4,7,8-pentachlorodibenzo-p-dioxin (4-PeCDF), octachlorodibenzo-p-dioxin (OCDD), 3,3',4,4'-tetrachlorobiphenyl (PCB77), 3,3',4,4',5-pentachlorobiphenyl (PCB126), and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB169). The mixture composition approximated the relative abundance of these compounds in foodstuff (L. S. Birnbaum and Tuomisto, 1994). Following the work of Gray et al. with TCDD (1997, Toxicology and Applied Pharmacology Vol. 146, pp. 11–20), we exposed time-pregnant dams on gestation day (GD) 15 at doses up to 1.0 μg TCDD/kg and the development of offspring was monitored. This mixture significantly increased the time to puberty in both male and female offspring. At postnatal day (PND) 32 seminal vesicle weights were decreased; however, only ventral prostate weight was affected at PND 49 and no effects were seen at PND 63. In female offspring, the mixture caused dose-dependent increases in the incidence of vaginal thread. Ethoxyresorufin-O-deethylase (EROD) activity was higher than with TCDD the comparable TEQ exposure. Based on the slightly lowered responsiveness to the mixture, we used 2.0 μg TEQ/kg to examine reproductive effects. This dose elicited the responses observed with 1.0 μg TCDD/kg. Results indicate that the mixture causes a similar spectrum of effects seen with TCDD and the slightly lowered degree of response based on administered dose appears to be due to decreased transfer of mixture components to the offspring. Thus, the use of the WHO consensus TEFs (M. Van den Berg et al., 1998, Environ. Health Perspec. 106, 775–792) reasonably predicts the developmental toxicity of this mixture of dioxin-like PHAHs.

Key Words: PHAH; TCDD; TEF; reproductive development.

Polyhalogenated aromatic hydrocarbons (PHAHs) are persistent bioaccumulative toxins (Safe, 1986). As a result of a variety of processes including combustion, chlorine bleaching, and, in the case of polychlorinated biphenyls (PCBs), commercial production, this class of compounds is found throughout the environment as complex mixtures. Exposures to laboratory animals and reports of human exposure after industrial accidents have demonstrated a variety of effects including wasting, chloracne, induction of xenobiotic metabolizing enzymes, and altered reproductive development (for reviews see Birnbaum, 1994; Birnbaum and Tuomisto, 2000; Pohjanvirta and Tuomisto, 1994). Recent work on the effects of PHAH compounds has demonstrated that low doses of the most toxic congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), administered to the dam during pregnancy results in altered reproductive development of both male and female offspring. Furthermore, these alterations occur in the rat (Gray et al., 1995; Gray and Ostby, 1995; Mably et al., 1992a), mouse (Theobald and Peterson, 1997), and hamster (Gray et al., 1995; Wolf et al., 1999). In addition, PCB77 and PCB126 (Faqi et al., 1998) and PCB169 (Gray et al., 1999) have been shown to alter male reproductive development in a similar manner to TCDD.

Due to the persistence of these compounds within the environment there is concern over the possible human health effects following exposure to dioxins. Much of this concern has focused on possible developmental effects, including alterations in reproduction and reproductive development. Taiwanese boys whose mothers ingested rice oil contaminated with PCBs and furans have smaller penises than did their age-matched control (Guo et al., 1995). Similarly, Gray et al. .
were used; 15 vehicle control and 15 dosed with 2.0 mg TEQ/kg. As a follow-up, 30 dams were treated by oral gavage on gestation day 15 using a dosing volume of 5 ml/kg. Sentinel animals were screened for sendai, rat coronavirus/sialodacryoadenitis (RCV/SDA), mycoplasma pulmonis, CARbacillus, parvovirus, Kilham rat virus (KRV), and pneumonia virus of mice.

### Determination of ethoxyresorufin O-deethylase (EROD) activity.
In order to define the extent of EROD induction, a sufficient number of GD 15 dams were exposed to 0.05, 0.2, 0.8, or 1.0 µg TEQ mixture or TCDD alone per kg so as to produce five litters per time point, per dosage level. Tissues were collected for determination of EROD activity on GD 21 and PND 4. From each litter at the time of sacrifice, maternal liver, a pool of 4 fetal or pup livers or a pool of 4 placenta, for GD 21, were collected and processed according to the method of DeVito et al. (1993, 1996). Briefly, tissues were homogenized in 10 volumes (w/v) of ice-cold phosphate-buffered saline, pH 7.4, using 5–7 strokes of a glass-teflon homogenizer. Homogenates were centrifuged at 9000 × g for 20 min and the resulting supernatant (S9) was collected, snap frozen in liquid nitrogen, and stored at −80°C.

For EROD measurement, S9 was thawed on ice, the supernatant filtered through cotton gauze, collected, and centrifuged at 100,000 × g for 1 h. The resulting microsomal pellet was resuspended in 400 ml PBS and used for EROD assays. Protein content of diluted microsomes was determined by the method of Bradford (1976) using BioRad protein assay reagents (Richmond, CA) and a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Bovine serum albumin was used as the standard.

EROD was determined by the method of Pohl and Fouts (1980) and Chaloupka et al. (1995) as modified by DeVito et al. (1993, 1996). Briefly, microsomal protein was diluted and added to 0.1 M K2HPO4, 5 mM MgSO4, and 2 mg bovine serum albumin/ml at pH 7.5 containing ethoxyresorufin (final concentration 1.5 µM). Samples were preincubated at 37°C and reactions initiated by the addition of 100 µl NADPH (5 mg/ml). Resorufin accumulation was monitored spectrofluorometrically with excitation and emission wavelengths of 522 and 586 nm, respectively.

### Collection of tissue for chemical analysis.
Animals used for chemical analysis were the same as those used to determine EROD activity with the exception that additional dams were dosed to provide tissues at GD 16. On GD 16, GD 21, and PND 4 maternal tissues collected included serum, liver, and adipose. Tissue collected from offspring included: all fetuses and placentas from GD 16 litters, all fetuses, except the four used for EROD measurements, on GD 21, and 1–2 pups per litter on PND 4. These samples were transported to Triangle Laboratories, Inc. (Durham, NC) on dry ice for later determination of the levels of TEQ mixture components in maternal and offspring tissues.

### Animal care and observation.
Dams were observed beginning on the morning of GD 21 until all dams had undergone parturition. On the day following the birth of the pups, anogenital distance (AGD) and body weight

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### Table 1

Dosing Solution Formulation

| TEQ TEQ fraction | TEQ | TEF | Ratio in food |
|------------------|-----|-----|--------------|
| TCDD             | 1.0 | 1.0 | 1            |
| TCD             | 1.0 | 1.0 | 1            |
| TCF             | 1.5 | 0.1 | 1.5          |
| T1-PeCDF         | 0.5 | 0.05| 0.025        |
| 2-PeCDF          | 2.0 | 0.5 | 1.0          |
| OCFD            | 5.0 | 0.0001| 0.0005    |
| PCB7           | 150 | 0.0001| 0.015      |
| PCB126         | 45  | 0.1  | 4.5          |
| PCBE99         | 30  | 0.01 | 0.3          |
| Total           | 7.9905|     |              |

*Van Birnbaum and DeVito, 1995.*

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**Chemicals.** TCDD, 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 2,3,7,8-tetrachlorodibenzo-p-dioxin (PeCDF), 1,2,3,4,7,8- and 1,2,3,4,6,7,8-TCDD, 1,2,3,7,8-pentachlorodibenzofuran (OCDF), TCD, 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF), 2,3,4,7,8-pentachlorodibenzofuran (5-PeCDF), 2,3,4,7,8-pentachlorodibenzofuran (TCDF), 1,2,3,7,8-pentachlorodibenzofuran (OCDF) were obtained from Ultra Scientific (North Kingstown, RI; purity > 98%). 3,3',4',4'-Tetrachlorobiphenyl (PCB77), 3,3',4',4'-pentachlorobiphenyl (PCB126), and 3,3',4',5',5'-hexachlorobiphenyl (PCB169) were obtained from Accustandard (New Haven, CT; purity > 99%).

To prepare the dosing solution, individual chemicals were dissolved in acetone, transferred to a defined volume of corn oil, and the acetone removed using a Savant Speed-Vac (Savant Instruments Inc., Farmingdale, NY). The composition of the dosing mixture (Table 1) was the product of the consensus mammalian TEF values (Van Birnbaum et al., 1998) and the ratio of these compounds in foodstuff (Birnbaum and DeVito, 1995). The concentration of chemicals within the final dosing solution was analyzed by high-resolution gas chromatography/high resolution mass spectrometry and reported elsewhere (Chen et al., 2001).

**Animals and dosing.** Time-pregnant Long Evans rats (gestation day [GD] 9; where the day after mating = GD 0) were obtained from Charles River Breeding Laboratories (same source as Gray et al., 1997, Raleigh, NC). Also as in the study of Gray et al. (1997a) females were housed in plastic cages containing heat-treated pine shavings (Beta Chips, NorthEastern Products Inc., Warrensburg, NY) and given food (Purina 5001 Rodent Chow,Ralston Purina Co., St. Louis, MO) and water *ad libitum.* Seventy-five dams were used with 15 dams dosed at 0, 0.05, 0.2, 0.8, or 1.0 µg TEQ/kg. As a follow-up, 30 dams were used; 15 vehicle control and 15 dosed with 2.0 µg TEQ/kg. All dams...
were recorded for all pups. On PND 4, litters were standardized by culling to five males and three females. Body weights and AGD were subsequently recorded for all remaining pups on PNDs 8, 15, and 22. At weaning, animals were housed as above in unisex groups of two to three rats/cage. Beginning on day 28, female pups were observed for vaginal opening and body weight recorded when a pinhole size or larger opening was first observed. Similarly, male pups were observed from PND 36 on for preputial separation and body weight recorded on the day of separation.

**Animal necropsies.** One male pup per litter (n = 10) was necropsied on PND 49 and 63. Whole body weight along with the weight of the liver, paired kidneys, paired adrenals, spleen, paired seminal vesicles with attached coagulating glands and their fluid content, ventral prostate, paired epididymides, and paired testis were recorded. The left cauda epididymis was removed from each animal, weighed, and used to determine cauda epididymal sperm counts. For the second and third exposures, male offspring were also necropsied on PND 32.

On PND 70 female offspring (n = 10/ dose group) were necropsied and body, liver, paired kidney, paired adrenal, spleen, and paired ovary weights were recorded. In addition, the offspring were examined for vaginal thread or evidence of cleft phallus using a dissecting microscope.

**Sperm counts.** Cauda epididymides were removed from PND 63 animals, weighed, and minced in a weight boat. Warm saline (1 ml) was added, mixed with tissue and the mixture transferred to a glass tube. An additional 1 ml of saline was used to rinse the weight boat and the entire mixture was incubated for 15 min at 37°C in the water bath. The incubation was ended by addition of 0.1 ml of 50% glutaraldehyde, the tube covered and placed in the refrigerator until counting. Sperm numbers were determined by diluting the samples and manually counting complete sperm (head attached to tail) using a hemocytometer and light microscope.

**Chemical analysis.** Four dams and their offspring were euthanized at GD 16, GD 21, or PND 4 to determine the distribution of mixture components. The analytical methods used are presented elsewhere (Chen et al., 2000). Briefly, tissue was ground in anhydrous sodium sulfate, sonicated with acetonitrile, and extracted using C18 solid-phase extraction cartridges. Extracts were cleaned up and analyzed using high resolution gas chromatography. Using the distribution data, we converted the concentration of individual chemicals into total tissue TEQ, using the WHO consensus TEF values (Van den Berg et al., 1998), in order to compare with data on the disposition of TCDD (Hurst et al., 2000). In addition, we compared the tissue distribution of individual chemicals in the mixture to the distribution of TCDD. To make the comparison with TCDD, we divided the dose of a given congener determined within fetal or neonatal tissue by the percent dose of TCDD within the tissue. Using these calculations, a congener that deposited within offspring with equal efficiency to TCDD would give a final ratio of 1; less efficient transfer would yield a ratio less than 1; more efficient transfer yields a ratio greater than 1.

**Statistics.** For necropsies throughout the study, one animal was used per litter. For other observations involving multiple animals from a given litter, litter means were used for statistical comparison. Levels of statistical significance were analyzed by ANOVA using StatView 512+ (Abacus Concepts, Berkeley, CA) for the Macintosh, followed by a Fisher PLSD-test as a post hoc test to compare means between different treatment groups. Differences were considered significant if p < 0.05.

## RESULTS

### Maternal Weight and Early Pup Mortality

The mixture did not affect maternal weight gain during pregnancy or number of live pups (Table 2). However, approximately 20% of pups died between PNDs 8 and 15, although there was no effect of treatment either on time of death or on percent mortality up to 1.0 μg/kg. In contrast, 2.0 μg TEQ/kg significantly (p < 0.05) increased mortality to 35% from approximately 20% in the concurrent controls. As with the earlier exposure, pups primarily died between PNDs 8 and 15.

## EROD Induction

EROD was determined at day 21 of gestation (Table 3) and postnatal day 4 (Table 4) in maternal and fetal/pup tissue. Maternal hepatic EROD was highest at GD 21 and no differences were noted between dams administered TCDD alone or the TEQ mixture across the dose range tested. In contrast by PND 4, hepatic EROD activity in dams administered TCDD alone was significantly greater. For example, at a dose of 1.0 μg TCDD/kg, activity was 718 ± 19 pmol/min/mg versus 176 ± 18 pmol/min/mg at the equivalent TEQ mixture dose. It appears the TEQ mixture dams have lower fold induction at PND 4 because of a greater decrease in EROD activity from 35.0 to 26.7 pmol/min/mg versus 176 pmol/min/mg at the equivalent TEQ mixture dose.

### TABLE 2

| Parameter                        | Control 1 | Control 2 | 0.05 | 0.2 | 0.8 | 1.0 | 2.0 |
|----------------------------------|-----------|-----------|------|-----|-----|-----|-----|
| Maternal wt. gain (g)            | 97.0 ± 6.0| 101.3 ± 7.6| 98.9 ± 5.0| 108.2 ± 4.0| 97.6 ± 8.7| 103.5 ± 4.3| 100.8 ± 6.1|
| Average litter size              | 12.9 ± 1.8| 12.3 ± 2.3| 12.0 ± 1.8| 14.0 ± 2.6| 13.2 ± 2.5| 12.4 ± 1.6| 12.8 ± 2.6|
| Mean pup wt. (g)                 | 6.5 ± 0.7 | 6.3 ± 0.7 | 6.6 ± 0.7 | 5.9 ± 0.4 | 6.2 ± 0.6 | 6.0 ± 0.6 | 6.1 ± 0.8 |

Note. Maternal weight gain was calculated from GD 9 to GD 20. Values are litter means ± SD.

*Control 2 was run simultaneously with the 2.0 μg TEQ/kg group.
was substantially higher in offspring. At the 0.8 and 1.0 doses, differences were not significant. By PND 4, enzyme activity was two- to threefold higher in TCDD-exposed tissue although statistically significant. Similarly, placental EROD activity was also significantly higher at GD 21 in TCDD-exposed fetuses and placenta.

In general, fetal/pup hepatic EROD exhibited a much larger fold induction than in the maternal hepatic microsomes. For example, at GD 21 maternal induction with 1.0 μg TCDD/kg was 33.5-fold versus 29.3-fold with the equivalent dose of the mixture. In contrast, within the same treatment groups, fetal induction was 155.9- and 183.1-fold. One reason for the high induction was the low levels of fetal control activity. Due to this low activity the data may be more appropriately evaluated using absolute activity values. Using this criteria, significantly higher EROD was seen at GD 21 in TCDD-exposed fetuses at the 0.8 μg/kg dosage and a nearly 2-fold increase at the 1.0 μg/kg dose, although this later difference was not statistically significant.

Puberty (Table 5) in female offspring (vaginal opening) was delayed 1.4 days at 0.8 μg/kg and 1.8 days at 1.0 μg/kg. At the 2.0 μg TEQ/kg dose, vaginal opening was delayed from 32.4 ± 0.3 to 32.2 ± 0.3; (p = 0.076). The time to puberty for the control females for the high dose exposure was significantly longer (p < 0.05) than the control group used in the dose-response exposure. Similarly, preputial separation in male offspring was delayed 1.6 days at 0.8 μg/kg and 1.7 days at 1.0 μg/kg. In addition, 2.0 μg TEQ/kg delayed puberty in male offspring an average of 2 days from 40.1 ± 0.3 days in controls to 42.1 ± 0.4 (p < 0.001).

Tissue Weights

At PND 32, a small number of male offspring (five to seven from different litters/dosage group) were necropsied and seminal vesicle weights determined (Table 6). Male offspring from litters exposed to 0.2 μg TEQ/kg and higher displayed significantly smaller seminal vesicle weights. At PND 49 (Table 7),

| Table 3 | EROD Activity in Maternal and Fetal Hepatic and Placental Microsomes at GD 21 |
|--------|---------------------------------|-----------------|-----------------|
| Dose μg/kg | TCDD TEQ mixture | TCDD TEQ mixture | TCDD TEQ mixture |
| Control  | 30.5 ± 5.4 | 40.3 ± 4.0 | 0.6 ± 0.6 | 0.3 ± 0.2 | 0.23 ± 0.14 | 0.24 ± 0.11 |
| 0.05     | 69.19 ± 6.6 | 76.8 ± 6.3 | 0.9 ± 0.3 | 0.5 ± 0.2 | 0.56 ± 0.17 | 0.16 ± 0.03*** |
| 0.2      | 390.8 ± 41.5 | 281.0 ± 22.8 | 1.9 ± 0.5 | 1.0 ± 0.5 | 0.68 ± 0.36 | 0.53 ± 0.31 |
| 0.8      | 794.7 ± 53.9 | 843.0 ± 8.4 | 67.3 ± 10.0 | 18.2 ± 1.2** | 2.74 ± 1.46 | 0.88 ± 0.31 |
| 1.0      | 1021.0 ± 109.4 | 1181.4 ± 108.0 | 97.9 ± 31.1 | 53.1 ± 14.1 | 1.74 ± 0.74 | 0.72 ± 0.09 |

Note. Units of activity are pmol/min/mg protein.
*Values are litter means with four fetuses or placentas per litter (n = 5).
**Significantly different from equivalent TCDD dose p < 0.05.
***Significantly different from equivalent TCDD dose p < 0.001.

| Table 4 | EROD Activity in Maternal and Pup Hepatic Microsomes at PND 4 |
|--------|---------------------------------|-----------------|-----------------|
| Dose μg/kg | Maternal | Mixture | Pup | Mixture |
| Control  | 27.4 ± 5.0 | 44.6 ± 5.6*** | 5.7 ± 0.9 | 5.0 ± 0.5 |
| 0.05     | 51.9 ± 4.2 | 48.3 ± 4.2 | 109.3 ± 23.1 | 83.2 ± 25.0 |
| 0.2      | 248.4 ± 19.1 | 121.2 ± 10.4** | 421.5 ± 41.6 | 284.3 ± 42.3 |
| 0.8      | 662.9 ± 25.6 | 354.9 ± 37.1*** | 786.3 ± 58.3 | 483.6 ± 77.4* |
| 1.0      | 718.2 ± 19.2 | 175.8 ± 17.5*** | 856.7 ± 47.2 | 611.0 ± 103.2* |

Note. Units of activity are pmol/min/mg protein.
*Values are means (n = 5) ± SE.
**Values are litter means (n = 5) with four pups per litter ± SE.
***Significantly different from equivalent TCDD dose p < 0.05.
****Significantly different from equivalent TCDD dose p < 0.001.
the weights of a number of male reproductive tissues were unchanged with only the ventral prostate having significant decreases at doses of 0.2 μg/kg and above. However, by PND 63, sperm were detected within the cauda epididymis and exposure to the mixture caused a significant decrease in number (Table 9). Control offspring had an average of 47.4 ± 3.1 × 10⁶ sperm per cauda epididymis and numbers were decreased 28–34% at all doses. However, no significant differences existed between dose groups. Cauda epididymal sperm numbers were further reduced from 44.6 ± 2.5 × 10⁶ in controls to 24.7 ± 1.5 × 10⁶ at the 2.0 μg/kg dose, a reduction of 45%.

**Morphological Alterations in Female Offspring**

Female offspring exposed to the mixture displayed a permanent vaginal thread. The thread incidence was elevated at all doses of the mixture (Table 10), showed a dose response increase and was significantly higher at 0.2 μg/kg (44%, p < 0.05), 0.8 μg/kg (73%, p < 0.001), and 1.0 μg/kg (83%, p < 0.001). Vaginal threads remained in offspring necropsied at PND 70.

At the 2.0 μg TEQ/kg dose, 60% of female offspring had cleft phallus of varying severity. In the most severe cases, a large opening in the urethra was found at the base of the phallus. Cleft phallus was not seen at lower doses.

**Chemical Disposition**

Tissue disposition of the mixture components is presented elsewhere (Chen et al., 2001). However, we were interested in examining disposition in offspring of individual congeners within the mixture relative to the transfer of TCDD (Table 11). Proportionally lower amounts of most congeners were detected in offspring; this was especially true of TCDF, 1-PeCDF, 4-PeCDF, OCDF, and PCB77. PeCDD, PCB126, and PCB169 were lower in fetal tissues, but their accumulation was similar to that of TCDD in PND 4 pups. This limited distribution to offspring resulted in fetal tissue TEQ concentrations that were approximately one third at GD 16 and two thirds at GD 21 that reported for TCDD alone (Table 12).
**TABLE 7**

Dose-Response Analysis of Tissue Weights of Male Offspring at PND 49

| Tissue   | Control 1 | Control 2* | 0.05 | 0.2  | 0.8  | 1.0  | 2.0  |
|----------|-----------|------------|------|------|------|------|------|
| Body     | 276 ± 9   | 310 ± 12   | 274 ± 12 | 263 ± 9 | 271 ± 8 | 269 ± 13 | 279.4 ± 7.5* |
| Liver    | 15.65 ± 0.50 | 20.39 ± 0.88 | 15.72 ± 1.67 | 14.82 ± 0.72 | 16.17 ± 0.78 | 15.94 ± 0.98 | 16.98 ± 1.73* |
|          | (65.80 ± 3.00)* |          |        |        |        |        | (62.44 ± 2.12)* |
| Kidney   | 2.94 ± 0.09 | 3.23 ± 0.09 | 2.91 ± 0.17 | 2.78 ± 0.08 | 2.86 ± 0.11 | 2.95 ± 0.15 | 3.04 ± 0.12 |
| Adrenals | 0.05 ± 0.002 | 0.050 ± 0.003 | 0.05 ± 0.002 | 0.05 ± 0.003 | 0.05 ± 0.003 | 0.05 ± 0.002 | 0.05 ± 0.002 |
| Spleen   | 0.76 ± 0.06 | 0.90 ± 0.04 | 0.85 ± 0.05 | 0.81 ± 0.02 | 0.78 ± 0.09 | 0.88 ± 0.03 | 0.84 ± 0.05 |
| SV tissue| 0.31 ± 0.02 | 0.33 ± 0.02 | 0.28 ± 0.02 | 0.30 ± 0.02 | 0.26 ± 0.02 | 0.29 ± 0.02 | 0.22 ± 0.02** |
| SV fluid | 0.13 ± 0.01 | 0.16 ± 0.01 | 0.10 ± 0.01 | 0.11 ± 0.02 | 0.09 ± 0.02 | 0.12 ± 0.01 | 0.10 ± 0.02* |
| SV       | (1.07 ± 0.07) | (0.53 ± 0.03) |        |        |        |        | (0.35 ± 0.06)* |
| Total SV | 0.43 ± 0.02 | 0.49 ± 0.24 | 0.38 ± 0.03 | 0.41 ± 0.03 | 0.36 ± 0.03 | 0.40 ± 0.03 | 0.32 ± 0.04** |
| Prostate | 0.15 ± 0.01 | 0.17 ± 0.01 | 0.12 ± 0.01 | 0.11 ± 0.01** | 0.09 ± 0.01** | 0.11 ± 0.01* | 0.08 ± 0.01*** |
| Epidid.  | 0.36 ± 0.03 | 0.37 ± 0.01 | 0.31 ± 0.01 | 0.31 ± 0.02 | 0.32 ± 0.01 | 0.31 ± 0.01 | 0.32 ± 0.01* |
| Testis   | 2.64 ± 0.14 | 2.78 ± 0.12 | 2.60 ± 0.07 | 2.60 ± 0.12 | 2.50 ± 0.10 | 2.44 ± 0.08 | 2.37 ± 0.10* |

Note. Values represent means ± SE (n = 8–10 males, each from different litters). All weights expressed in grams.

*aControl 2 was run simultaneously with the 2.0 µg TEQ/kg group.

Numbers in parentheses are organ wt./kg body weight.

**p < 0.05.

***p < 0.001.

**DISCUSSION**

Dioxin-like PHAHs are known to alter reproductive development of laboratory animals in a dose-response manner (Gray et al., 1997a; Mably et al., 1992a). In the current study, a mixture of dioxins, furans, and non-coplanar PCBs produced a similar spectrum of developmental reproductive alterations as seen in exposures to TCDD alone, i.e., decreased sperm counts, delays in puberty, vaginal thread and decreased accessory gland weight. However, two to three times higher administered doses of the current mixture appeared necessary to elicit many of the alterations and less sensitive endpoints, such as clefthallus, which were only seen at a dose of 2.0 µg TEQ/kg.

**TABLE 8**

Tissue Weights of Male Offspring at PND 63

| Tissue   | Control | 2.0 µg TEQ/kg |
|----------|---------|---------------|
| Body     | 419.3 ± 7.0 | 404.1 ± 10.9 |
| Liver    | 25.26 ± 0.74 | 24.68 ± 1.17 |
| Kidney   | 3.90 ± 0.14 | 4.10 ± 0.20 |
| Adrenals | 0.058 ± 0.0041 | 0.057 ± 0.0044 |
| Spleen   | 0.98 ± 0.079 | 0.94 ± 0.053 |
| SV tissue| 0.53 ± 0.022 | 0.44 ± 0.019** |
| SV fluid | 0.37 ± 0.025 | 0.28 ± 0.020* |
| Total SV | 0.90 ± 0.032 | 0.72 ± 0.035*** |
| Prostate | 0.27 ± 0.016 | 0.18 ± 0.011*** |
| Epidid.  | 0.73 ± 0.027 | 0.70 ± 0.022 |
| Testis   | 3.40 ± 0.17 | 2.89 ± 0.30* |

Note. Values represent means ± SE (n = 10 males, each from separate litters). All weights are expressed in grams.

*p < 0.05.

**p < 0.01.

***p < 0.001.

**TABLE 9**

Cauda Epididymal Sperm Counts at PND 63

| Dose µg TEQ/kg | Cauda epididymal sperm (× 10⁶) |
|---------------|-----------------------------|
| Control 1     | 47.4 ± 3.1 |
| Control 2*    | 44.6 ± 2.2 |
| 0.05          | 34.2 ± 1.3* |
| 0.2           | 30.6 ± 1.4** |
| 0.8           | 28.1 ± 1.4** |
| 1.0           | 31.3 ± 1.1* |
| 2.0           | 24.7 ± 1.3*** |

Note. Values represent means ± SE from 8–10 males from different litters.

*aControl 2 was run simultaneously with the 2.0 µg TEQ/kg group.

*p < 0.05.

**p < 0.01.

***p < 0.001.
Previous exposures of pregnant dams to TCDD have reported that epididymal sperm counts and vaginal thread are among the most sensitive measures of effect in male and female offspring, respectively (Gray et al., 1997b; Mably et al., 1992b). Changes in the weights of the accessory glands of the male reproductive tract are a less sensitive measure although sensitivity depends on the time of development at which measurements are taken. In general, the magnitude of the effect is greatest as the animal approaches puberty and tends to recover at later timepoints. For example, the seminal vesicles from in utero and lactationally exposed offspring display the greatest difference from controls at PND 32 (Hamm et al., 1992b). Changes in the weights of the accessory glands of the male offspring, respectively (Gray et al., 1997b; Mably et al., 1992b) reported a 30% decrease in cauda epididymal sperm of PND 63 offspring. Furthermore, they noted that cleft phallus, a high dose effect contrast, Mably et al. (1992b) reported decreases in cauda epididymal sperm of PND 63 male Holtzman rat offspring at doses as low as 0.064 μg/kg TCDD, and as much as a 75% decrease at 1.0 μg/kg TCDD. In the current study, epididymal sperm counts were a sensitive measure of effect. While the magnitude of response was not as great as those reported by Mably et al. (1992b), significant effects were seen at the lowest dose tested, 0.5 μg/kg. In contrast, Faqi et al. (1998) reported increased daily sperm production following exposure to 100 μg PCB77/kg, while 10 μg PCB126/kg did not affect sperm counts. However, Faqi et al. also reported to 2.0 μg TEQ/kg. Similarly, cleft phallus, a high dose effect (Gray et al., 1997b), was only seen at 2.0 μg TEQ/kg.

Gray et al. (1997a) did not report any effect on testicular sperm counts in Long Evans rat offspring. Furthermore, they reported a 30% decrease in cauda epididymal sperm of PND 63 offspring only at the high dose used; 0.8 μg/kg TCDD. In contrast, Mably et al. (1992b) reported decreases in cauda epididymal sperm of PND 63 male Holtzman rat offspring at doses as low as 0.064 μg/kg TCDD/kg, the low dose tested, and as much as a 75% decrease at 1.0 μg/kg TCDD. In the current study, epididymal sperm counts were a sensitive measure of effect. While the magnitude of response was not as great as those reported by Mably et al. (1992b), significant effects were seen at the lowest dose tested, 0.5 μg/kg. In contrast, Faqi et al. (1998) reported increased daily sperm production following exposure to 100 μg PCB77/kg, while 10 μg PCB126/kg did not affect sperm counts. However, Faqi et al. also reported

### Table 10

| Dose μg TEQ/kg | Percentage of females with vaginal thread |
|---------------|------------------------------------------|
| Control 1     | 3 ± 3                                    |
| Control 2*    | 16 ± 4                                   |
| 0.05          | 23 ± 10                                  |
| 0.2           | 44 ± 13*                                 |
| 0.8           | 73 ± 10***                               |
| 1.0           | 83 ± 9***                                |
| 2.0           | 78 ± 10***                               |

*Note. Values are litter means ± SE (n = 10+).

*Control 2 was run simultaneously with the 2.0 μg TEQ/kg group and had a significantly higher percentage of females with vaginal thread.

### Table 11

| Dose μg TEQ/kg | TCDF | PCDF | 1-PeCDF | 4-PeCDF | OCDF | PCB77 | PCB126 | PCB169 |
|---------------|------|------|---------|---------|------|-------|--------|--------|
| GD 16         |      |      |         |         |      |       |        |        |
| 0.05          | 0.08 ± 0.03 | 0.09 ± 0.01 | ND      | 0.03 ± 0.01 | ND   | 0.05 ± 0.05 | 0.72 ± 0.04 | 0.68 ± 0.03 |
| 0.2           | 0.08 ± 0.02 | 0.11 ± 0.02 | 0.02 ± 0.02 | 0.04 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.49 ± 0.07 | 0.58 ± 0.08 |
| 0.8           | 0.11 ± 0.02 | 0.16 ± 0.05 | 0.02 ± 0.01 | 0.04 ± 0.02 | 0.003 ± 0.001 | 0.01 ± 0.003 | 0.37 ± 0.07 | 0.49 ± 0.11 |
| 1.0           | 0.15 ± 0.03 | 0.28 ± 0.06 | 0.11 ± 0.04 | 0.14 ± 0.03 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.29 ± 0.04 | 0.37 ± 0.05 |
| GD 21         |      |      |         |         |      |       |        |        |
| 0.05          | ND   | 0.36 ± 0.03 | ND      | 0.12 ± 0.01 | ND   | 0.01 ± 0.01 | 0.21 ± 0.04 | 0.18 ± 0.03 |
| 0.2           | 0.01 ± 0.02 | 0.47 ± 0.09 | ND      | 0.13 ± 0.04 | 0.01 ± 0.002 | 0.003 ± 0.002 | 0.25 ± 0.07 | 0.21 ± 0.06 |
| 0.8           | ND   | 0.36 ± 0.05 | 0.02 ± 0.003 | 0.09 ± 0.02 | 0.005 ± 0.002 | 0.003 ± 0.001 | 0.24 ± 0.02 | 0.24 ± 0.03 |
| 1.0           | ND   | 0.39 ± 0.15 | ND      | 0.11 ± 0.04 | 0.007 ± 0.002 | 0.003 ± 0.002 | 0.28 ± 0.09 | 0.27 ± 0.10 |
| PND 4         |      |      |         |         |      |       |        |        |
| 0.05          | 0.17 ± 0.03 | 0.81 ± 0.20 | 0.18 ± 0.02 | 0.29 ± 0.05 | 0.01 ± 0.003 | 0.02 ± 0.01 | 0.88 ± 0.11 | 0.75 ± 0.16 |
| 0.2           | 0.02 ± 0.01 | 0.80 ± 0.08 | 0.04 ± 0.02 | 0.19 ± 0.02 | 0.01 ± 0.004 | 0.001 ± 0.001 | 0.93 ± 0.11 | 0.97 ± 0.15 |
| 0.8           | 0.004 ± 0.003 | 0.76 ± 0.12 | 0.03 ± 0.002 | 0.16 ± 0.03 | 0.02 ± 0.009 | 0.0006 ± 0.0008 | 0.82 ± 0.15 | 0.98 ± 0.16 |
| 1.0           | 0.004 ± 0.0008 | 0.67 ± 0.14 | 0.03 ± 0.008 | 0.14 ± 0.03 | 0.02 ± 0.006 | 0.0004 ± 0.0002 | 0.75 ± 0.15 | 0.88 ± 0.18 |

*Note. ND = compound not detected. Tissue disposition data presented in Chen et al., 2001. Values were calculated as follows: the percent dose of the congener/the percent dose of TCDD. Values are means ± SD (n = 4).
enlarged testicles following exposure to PCB77, whereas our mixture decreased testicular size at the high dose. It is important to note that PCB 77, unlike persistent PCBs 126 and 169, is rapidly metabolized to reactive intermediates, which may have effects of their own (Pereg et al., 1999).

Pharmacokinetic differences between PHAH congeners exist and are known to influence their relative potency (DeVito and Birnbaum, 1995; DeVito et al., 1998). The decreased degree of responsiveness in EROD induction and developmental reproductive effects using the TEQ mixture suggests either slight conservatism of the TEFs and/or a lower tissue distribution of compounds within the mixture than would be expected with an equivalent dose of TCDD. Using the disposition data from this exposure (Chen et al., 2001), a comparison of the total toxic equivalency within offspring shows the mixture resulted in lower TEQ within the offspring than has been reported for equivalent administered doses of TCDD in our laboratory (Hurst et al., 2000). This was in part due to the limited transfer to the fetus of TCDD, 1-PeCDF, and PCB77 (Chen et al., 2001). TCDD, 1-PeCDF, and PCB77 are readily metabolized by CYP1A1 (Brewster and Birnbaum, 1988; Olson et al., 1994) and the induction of this enzyme by the mixture likely induced the metabolism and excretion of these congeners. However, as these three compounds only contribute approximately 2% of the TEQ in the current mixture their absence in offspring would not account for the decreased responsiveness.

In order to determine which compounds might account for the decreased toxicity relative to TCDD, we compared the disposition of the mixture components to the disposition of TCDD. Based upon their relative masses in the mixture and TEFs, the major contributors to the toxicity of the mixture should be TCDD, PeCDD, 4-PeCDF, and PCB126. Using a mass to mass comparison, TCDD was more readily transferred to fetuses than the other three compounds. In contrast, disposition of PeCDD, PCB126, and PCB169 were relatively similar to TCDD in offspring by PND 4, indicating greater lactational transfer of these three compounds. For example, TCDD concentrations increased in offspring 4- to 6-fold between GD 21 and PND 4, whereas PCBs 126 and 169 increased 13- to 25-fold over this same period. In contrast, 4-PeCDF remained relatively lower in offspring throughout the study period presumably due to the elevated levels in maternal liver. 4-PeCDF is known to be sequestered in liver to a greater extent than TCDD (DeVito et al., 1998). Hepatic sequestration is due to the induction and subsequent binding to CYP1A2 (Diliberto et al., 1999). As stated in Chen et al. (2001), PeCDD, 4-PeCDF, and PCB126 all had a greater percentage of the dose in maternal liver than TCDD. This sequestration in liver likely was a factor in the limited transfer to offspring.

Another important factor in the effects studied is the timing of exposure; i.e., the relationship between tissue concentrations and the critical window for effects. Alterations in the developing ventral prostate have been demonstrated histologically as early as GD 20 (Roman et al., 1998). Similarly, alterations in the vaginal tract preceding vaginal thread formation have been shown to occur during gestation (Dienhart et al., 2000; Hurst et al., 2001). From these observations it would appear that gestational exposure is critical and therefore fetal tissue concentrations would parallel the magnitude of effect. However, Bjerke and Peterson (1994) demonstrated that in addition to in utero exposure, exposure of offspring only by lactational transfer of TCDD was capable of altering the development of the ventral prostate and seminal vesicles. Furthermore, the cumulative impact of in utero and lactational exposure on the development of the prostate was greater than when exposure was restricted to the prenatal period alone. A clearer understanding of the critical window of development is crucial in the interpretation of the correlation between tissue levels and degree to which development is affected.

In conclusion, administered TEQ dose was a reasonable predictor of the reproductive developmental effects studied; the dose of the current mixture affecting most endpoints was within a factor of two of the equivalent TCDD dose. Analysis of the disposition data demonstrates that mixture components were not as readily transferred to offspring as TCDD. These pharmacokinetic differences resulted in lowered tissue TEQ within offspring and likely underlie the decreased toxicity of the mixture. The TEF approach assumes that the mechanism of action involves binding to and activation of the arylhydrocarbon receptor (AhR; Birnbaum, 1999; Van den Berg et al., 1998). Studies in AhR–/– mice have shown that the absence of this protein eliminates the developmental toxicity of TCDD (Mimura et al., 1997; Peters et al., 1999). Since the toxicity of the current mixture was reasonably predicted through the use of TEFs, this further supports the hypothesis that the reproductive alterations involve an AhR mediated mechanism.

Future work should examine the effects of non-dioxin-like PCBs on reproductive development both alone and in combination with additional mixtures of dioxin-like congeners. The pharmacokinetic differences in transfer from the dam to offspring that exist between TCDD and other components of this mixture deserve additional attention.

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REFERENCES

Bjerke, D. L., and Peterson, R. E. (1994). Reproductive toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male rats: Different effects of in utero versus lactational exposure. Toxicol. Appl. Pharmacol. 127, 241–249.
Brewster, D. W., and Birnbaum, L. S. (1988). Disposition of 1,2,3,7,8-penta-
Birnbaum, L. S. (1994). Endocrine effects of prenatal exposure to PCBs,
Birnbaum, L. S. (1994). Endocrine effects of prenatal exposure to PCBs,
Birnbaum L. S., and Tuomisto, J. (2000). Non-cancer effects of dioxins.
Birnbaum, L. S. (1998). Dose-response relationships for disposition and
Gray, L. E., Kelce, W. R., Monosson, E., Ostby, J. S., and Birnbaum, L. S.
Gray, L. E., Kelce, W. R., Monosson, E., Ostby, J. S., and Birnbaum, L. S. (1995). Exposure to TCDD during development permanently alters repro-
ductive function in male Long Evans rats and hamsters: Reduced ejaculated
and epidydmal sperm numbers and sex accessory gland weights in offspring
with normal androgenic status. Toxicol. Appl. Pharmacol. 131, 108–118.
Gray, L. E., and Ostby, J. S. (1995). In utero 2,3,7,8-tetrachlorodibenzo-p-
dioxin (TCDD) alters reproductive morphology and function in female rat
offspring. Toxicol. Appl. Pharmacol. 133, 285–294.
Gray, L. E., Ostby, J. S., and Kelce, W. R. (1997a). A dose-response analysis
of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlo-
rodibenzo-p-dioxin in male Long Evans hooded rat offspring. Toxicol. Appl. Pharmacol. 146, 11–20.
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gr
dose-response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlo-
rodibenzo-p-dioxin in male Long Evans hooded rat offspring. Toxicol. Appl. Pharmacol. 146, 11–20.
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Gray, L. E., Wolf, C., Mann, P., and Ostby, J. S. (1997b). In utero exposure to low
doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive develop-
ment of female Long Evans rats offspring. Toxicol. Appl. Pharmacol. 146,
Peters, J. M., Narotsky, N. G., Elizondo, G., Fernandez-Salguero, P. M., Gonzalez, F. J., and Abbott, B. D. (1999). Amelioration of TCDD-induced teratogenesis in aryl hydrocarbon receptor (AhR)-null mice. Toxicol. Sci. 47, 86–92.

Pohjanvirta, R., and Tuomisto, J. (1994). Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: Effects, mechanisms, and animal models. Pharmacol. Rev. 46, 483–549.

Pohl, R. A., and Fouts, J. R. (1980). A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Anal. Biochem. 107, 150–155.

Roman, B. L., Timms, B. G., Prins, G. S., and Peterson, R. E. (1998). In utero and lactational exposure of the male rat to 2,3,7,8-tetrachlorodibenzo-p-dioxin: Impairs prostate development 2. Effects on growth and cytodifferentiation. Toxicol. Appl. Pharmacol. 150, 254–270.

Safe, S. H. (1986). Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Annu. Rev. Pharmacol. Toxicol. 26, 371–79.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit. Rev. Toxicol. 21, 51–88.

Safe, S. H. (1994). Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev. Toxicol. 24, 87–149.

Theobald, H. M., and Peterson, R. E. (1997). In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: Effects on development of the male and female reproductive system of the mouse. Toxicol. Appl. Pharmacol. 145, 124–135.

Van den Berg, M., Birnbaum, L. S., Bosveld, A. T. C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., et al. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Perspect. 106, 775–792.

Van den Berg, M., Heeremans, C., Veenhoven, E., and Olie, K. (1987). Transfer of polychlorinated dibenzo-p-dioxins and dibenzofurans to fetal and neonatal rats. Fundam. Appl. Toxicol. 9, 635–644.

Wolf, C. J., Ostby, J. S., and Gray L. E. (1999). Gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female hamster offspring. Toxicol. Sci. 51, 259–264.