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The Effects of Chlordane Exposure during Pre- and Postnatal Periods at Environmentally Relevant Levels on Sex Steroid-Mediated Behaviors and Functions in the Rat[1]

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Technical chlordane is a mixture of four main isomers (i.e., heptachlor, *cis*-chlordane, *trans*-chlordane, and *trans*-nonachlor) found in meat and dairy products as well as in indoor air of houses treated for termites. These isomers are metabolized to more potent epoxides (heptachlor epoxide and oxychlordane) which accumulate in lipid compartments of tissues and have been shown to reduce chloride influx through $GABA_A$ receptor complex channels and to alter steroid levels. However, considering the almost universal human exposure and the potential for accumulation of these agents, very little is known about how chronic, low-level exposures during development affect adult behavior and steroid-mediated processes. Time-pregnant Sprague–Dawley dams (Day 4 of gestation through Day 21 of lactation) and offspring (Day 22 of age through Day 80) were exposed to three levels of technical chlordane (100, 500, or 5000 ng/g) on a daily schedule. The low-exposure level generated heptachlor epoxide and oxychlordane plasma levels in the dam (Day 20) and in the offspring (Day 80) representative of those found in the U.S. populace. Chlordane-dosed offspring exhibited sex- and dose-dependent effects on testosterone levels, behavioral tests, and body weight conducted between postnatal Days 77 and 85. Chlordane-dosed females, but not males, had significant decreases in testosterone levels, significant improvements in spatial abilities (i.e., decreases in Cincinnati maze errors, navigation times, and failures to escape), and significant increases in body weight and in auditory startle-evoked responses. In two other tests, only males were used. These chlordane-dosed males showed significant increases in male-typical mating behaviors and decreases in $^{36}Cl^-$ uptake into brain microsacs. For all behavioral and body weight measurements, dose–response effects were observed for the 100 and 500 ng/g dosed groups. However, the 5000 ng/g dose group responses were closer to those of control values. These results suggest that these cyclodienes masculinize sexually dimorphic functions and behaviors by mimicking sex steroids and/or changing their levels.

Technical chlordane is a mixture (80% by weight) of four cyclodienes, heptachlor, *cis*- and *trans*-chlordane, and *trans*-nanochlor, having the same cyclodiene carbon skeleton substituted with 7, 8, and 9 chlorines. During the 40 years that chlordane/heptachlor has been registered as an insecticide in the United States, over 250 million pounds have been used (Liddle, 1988). These cyclodienes were used extensively in agriculture in the United States during the 1950s, 1960s, and 1970s (MacMonegle *et al.*, 1984) and are still used in foreign countries. These cyclodienes are metabolized to epoxides which accumulate in soils (Lichtenstein *et al.*, 1970), in grains (MacMonegle *et al.*, 1984), and in lipid-containing compartments such as adipose tissue and milk (Tashiro and Matsumura, 1978; Hirasawa and Takizawa, 1989). Consistent levels (30–50 ng/g) of these epoxides, heptachlor epoxide and oxychlordane, have been reported in beef fat and cow milk fat from 1972 to 1990 (Steffey *et al.*, 1984; Salman *et al.*, 1990).

The other major route of human exposure is the inhalation of chlordane/heptachlor in indoor air of houses treated for termites. The Air Force sampled ambient air from 4700 housing units and reported detectable levels in 45%, of which 35% exceeded 2 $\mu g/m^3$ (USEPA, 1987). Even higher levels have been reported for privately owned houses. Leidy *et al.* (1985) sampled ambient air from 60 houses that had been treated within the past 5 years. The results for chlordane ranged from 0.05 to 9.9 $\mu g/m^3$. These values are an order of magnitude or more above EPA's reference dose (RfD, 0.05 $\mu g/kg/day$), which was based on hepatotoxicity data and equates to an ambient air concentration of 0.2 $\mu g/m^3$ (USEPA, 1987). When these levels of exposure are considered for the 30 million houses that have been treated with technical chlordane or technical chlordane mixed with

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heptachlor, the potential for health effects is evident (Liddle, 1988).

The use of technical chlordane as an agricultural insecticide and as a termiticide has resulted in consistent levels of oxychlordane and heptachlor epoxide in human lipid-containing compartments. National surveys conducted during the 1970s and 1980s have reported constant heptachlor epoxide and oxychlordane levels in human adipose tissues and human milk to be approximately 100 ng/g of lipid (Kutz *et al.*, 1991; Savage *et al.*, 1981). For comparison, the average level of tetrachlorodibenzodioxin (TCDD) in human fat during this period was 7.7 pg/g (Gross *et al.*, 1984). Thus, the levels of heptachlor epoxide and oxychlordane in human fat are approximately 10,000 times higher than the concentration of TCDD in human fat.

Exposure to cyclodienes during gestation and lactation appears to be greater than that during other periods of life and corresponds to the period of nervous system development. Levels of heptachlor epoxide in human fetal blood have been reported to be almost four times the levels in maternal blood (Polishuk *et al.*, 1977). Furthermore, high levels of heptachlor epoxide and oxychlordane have been reported in mothers' milk (Savage *et al.*, 1981). Exposure of American babies to cyclodiene epoxides during the developmental period is considerable and corresponds to the time when the CNS is undergoing sexually dimorphoric organization. This differentiation of the CNS is controlled by sex steroids and results in the sexually dimorphoric phenotypes.

Chlordane has been reported to decrease levels of testosterone, estradiol, deoxycorticosterone, and progesterone in various body compartments by increasing levels of the steroid hydroxylase (Haake *et al.*, 1987; Levin *et al.*, 1968; Welch *et al.*, 1971; Conney *et al.*, 1966). Chlordane has also been reported to reduce estradiol-induced uterine weight and testosterone-induced seminal vesicle weight (Welch *et al.*, 1971; Levin *et al.*, 1969). Furthermore, decreased levels of gonadotropins (FSH and LH) in sera of mature rats have been reported after exposure to chlordane and dieldrin, another cyclodiene (Wedig and Vernon, 1973; Ateia *et al.*, 1990). Finally, chlordane (0.16 and 8.00 μg/g) has been shown to alter corticosterone levels in plasma at 101 and 400 days of age in mice dosed throughout gestation (Cranmer *et al.*, 1984). Taken together, these studies suggest that these cyclodienes are mimicking endogenous steroids. If these cyclodienes are mimicking steroids, then low exposures during nondevelopmental periods could have transient behavioral effects. However, if exposure occurs during a critical developmental period(s), then the offspring could be permanently altered in their reactions to stress or on a variety of sexually dimorphic behaviors/phenotypes (e.g., body weight, development of sexual organs, circulating steroid levels, mating behavior, spatial abilities, activity level, or mixed function oxidase levels). This study assessed a variety of endpoints in rat offspring exposed to environmentally relevant levels of technical chlordane during pre- and postnatal periods.

METHODS

Subjects

Sprague–Dawley CD time-pregnant rats were shipped on the second day of pregnancy from Charles River Laboratories Inc. (Portage, MI) and placed in individual polycarbonate rearing cages on the third day of gestation. Pregnant dams and dams with offspring were housed in the standard rearing cages until pups reach 22 days of age. Litter sizes were culled to 10 offspring, 5 of each sex when possible, by Postnatal Day (PND) 4. To increase the probability of obtaining 5 offspring of each sex, twice as many timed-pregnant rats were purchased as needed for each replicate. This selection of litters with approximate equal number of each sex may have reduced the variance of sex-steroid-mediated behaviors. Previous reports have demonstrated masculinization of female offspring from litters containing more males than females. From PND 22 to 80, pups were housed separately in stainless steel cages. Rats were maintained on a 12-hr light/12-hr dark cycle, with NIH 07 Rodent-assay wafer (Zeigler Brothers, Inc.) and filtered tap water was available *ad libitum* in animal facilities certified by the American Association for Accreditation of Laboratory Animal Care.

Treatment

Rats were dosed (w/w) daily using peanut oil supplemented with and without technical chlordane spiked into 1 cc of peanut butter (Cranmer *et al.*, 1978). Using this dosing protocol a control and three dose groups were prepared: 100, 500, and 5000 ng/g. Stocks were prepared by mixing technical chlordane (Lot 30–54, Chem Service Inc.) into peanut oil at three stock concentrations: 2, 10, and 100 μg/μl. Dosing volume was based on average body weight calculated twice a week for each dose group of dams and each dose group and sex of offspring. Dams were dosed from Day 4 of gestation until Day 21 of lactation. Pups were dosed individually from PND 22 to 80. From earlier pilot studies, the low-dose group (100 ng/g) generated heptachlor epoxide and oxychlordane levels in the dam (Day 20) and in the offspring (PND 80) representative of serum levels found in the United States at the 99th percentile (i.e., 1% of the U.S. values higher). The two high-dosed groups were fed technical chlordane 5 and 50 times the low-dose level.

Offspring were tested in the Cincinnati maze beginning on PND 76 in the open field activity (OF) and auditory startle (AS) paradigms beginning on PND 81. Male offspring only were evaluated for various male-typical mating behaviors on PND 77 and the brains of high-dose males were used on PND 85 to conduct Cl^- uptake studies.

Collecting and Analyzing Cyclodienes in Rat Blood and Milk

Collection of blood samples. Blood was collected from dams on Day 2, 7, 13, and 20 of gestation and on Day 6 and 18 of lactation, and from offspring on PND 41, 61, and 81. Dams and offspring were placed head-first into the apex of plastic decapitation cones (Harvard Apparatus Inc.). The base of the cone was tied around the tail with a latex glove and the rat was suspended by the cone apex using the polycarbonate cage as a support. The tail was placed in a vessel containing warm water for approximately 2 min. The suspension and warm water dilated the tail veins which were cut perpendicular to the long axis of the tail. Approximately 200–240 μl of blood was collected in heparinized Natelson Blood Collecting Tubes Red (Fisher). The collecting end was sealed with Tube Sealing Compound (Cha-Seal).

Plasma was separated from cellular components by centrifugation for 10 min at 900*g*. The top end was subsequently sealed with sealing compound and the sample was stored at −20°C.

Milk was collected from dams of litters not used in testing on Day 8 by

holding the dam on her back with one hand and milking the dam with the index finger and thumb of the other hand. Natelson Blood Collecting Tubes Red (250 μl) were used to collect and store the milk.

Sparging and analysis. Frozen blood sample tubes were scored and broken at the plasma–red blood cell interface and the plasma–air interface. The frozen plasma (50–100 μl) contained in the capillary tube was immediately placed in a sparging vessel containing 10 ml of purified water. Milk samples were handled in a similar manner except only 5–15 μl of milk was placed in the sparging vessel. In both cases, the solution was sparged with ultrapure nitrogen (99.999%) for 30 min at 120°C using a Envirochem Dynamic Stripper Model 1260. The analytes were collected in a sorbent tube containing Tenax GC resin (5 cm × 4 mm i.d.). The sorbent tube was then thermally desorbed (Envirochem Unacon Series 810) into a gas chromatograph (Hewlett Packard 5790) equipped with a 30-m, 0.25-mm-i.d. fused silica capillary column (SPB 608) (Supelco) and an electron capture detector. Peak areas were integrated with a Hewlett Packard 3390 integrator. Areas of peaks with retention times corresponding to heptachlor, heptachlor epoxide, and oxychlordane standards were compared with standard curves constructed with certified environmental standards (Ultra Scientific) spiked into rat plasma. *trans*-Chlordane retention time was determined from standards spiked directly into sparging water. Quantitation of *trans*-chlordane concentration in rat plasma was based on the oxychlordane standard curve.

Active Testosterone

Active testosterone was measured in rat serum collected on PND 85 by decapitation. Testosterone levels were determined using a wall-coated radioimmunoassay kit purchased from Diagnostic System Laboratories (Webster, TX). A testosterone standard, a control, or a rat serum sample (50 μl) was added to anti-testosterone IgG-coated tubes. Immediately, 500 μl of ^{125}I-labeled testosterone reagent was added to all tubes. Racks of tubes were gently shaken by hand for 10 sec and placed in a water bath at 37 ± 2°C for 65 min. After 65 min, all tubes except total count tubes were aspirated and then counted for 1 min in a gamma counter. Results were determined from a standard curve.

Body Weight

Dams and offspring were weighed twice a week at 3- and 4-day intervals throughout the study. Offspring were not weighed individually until after being weaned and relocated to individual cages on Day 22.

Neurobehavioral Endpoints

Cincinnati water maze. Each rat was tested in the Cincinnati maze paradigm as described by Vorhees (1987). The maze is composed of nine Ts with each T measuring 15.2 cm in stem length and 22.9 cm in each cul-de-sac arm. The maze wall height was 50.8 cm and the maze was filled with water to a depth of 25.2 cm. Water temperature was maintained at 21 + 1°C. Rats were tested in the maze using the following sequence: Day 1 consisted of three trials in a 150-cm straight channel as a test of swimming ability and motivation to escape from water; Days 2–5 consisted of two trials per day in path B, the more difficult of the two paths used with this test (Vorhees *et al.*, 1991). On all trials, navigation time for each was recorded. On all maze trials, errors for each rat were also recorded. An error was defined as a whole-body entry into any blind T. Entry into one arm of the T and then turning and entering the second arm prior to exiting was scored as two errors. Reentry into the start arm, once it had been exited, was also scored as an error. Rats were allowed 5 min to solve the maze and then were removed, thoroughly dried, and given a 1-hr rest before their second trial.

Mating behaviors. Male offspring were placed with primed females in a circular activity chamber 45.7 cm in diameter for 30 min on PND 77 and latency to first intromission, number of intromissions before ejaculation, total intromissions, and latency to first ejaculation were recorded. Females were brought into behavioral estrus by subcutaneous injections of 100 μg estradiol benzoate in 0.1 ml peanut oil approximately 48 hr prior to testing, followed by 1.0 mg of progesterone in 0.1 ml peanut oil 3–5 hr prior to testing. All testing was conducted within the first 4 hr of the dark cycle. The test area was illuminated with a 20-W red light bulb.

Open field activity. Each rat was tested in an open field activity paradigm on PND 81. The tests were conducted in a darkened room using an automated open field apparatus for 30 min. The open field apparatus was circular (45.7 cm in diameter) with four parallel photobeams aligned on the x axis and four on the y axis (San Diego Instruments, San Diego, CA). Single photobeam interruptions were scored as peripheral area movement and simultaneous x–y beam interruptions were scored as central movement.

Auditory startle. Each rat was tested in a startle reaction (auditory) paradigm on PND 81. The test was conducted for 51 trials using a SR startle chamber (San Diego Instruments) with an auditory startle signal of 110 dB lasting 20 msec. The intertrial interval (ITI) was 8 sec and the recording window was 100 msec after signal onset. The chamber was sound and light controlled and emitted a continuous background white noise level (4 kHz peak) of 72 dB to enhance the response. Responses measured were maximum response amplitude (V_{max}), average response amplitude (V_{ave}), and latency to maximum response (T_{max}).

Neurochemical Endpoint

^{36}Chloride uptake. Chloride-36 uptake into rat brain microsacs was determined using procedures described by Gant *et al.* (1987) with minor modifications. Control and 5000 ng/g male Sprague–Dawley rats 85 days of age were decapitated and their brains were rapidly removed and homogenized with a Teflon–glass homogenizer (five strokes, 1000 rpm) with a clearance of 0.025 in. in 7 ml ice-cold Mops buffer (133.0 mM NaCl, 5.0 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 7.0 mM NaOH, 1.0 mM ascorbic acid, 1.2 mM $CaCl \cdot 2H_2O$, 10.0 mM *d*-glucose, 16.3 mM 3-(4-morpholine)propanesulfonic acid (Mops) adjusted to pH 7.4 with HCl. The homogenate was centrifuged at 900*g* for 15 min, the supernate was decanted, and the pellet was resuspended in 20 ml of the same buffer and recentrifuged at 900*g* for 15 min. The final pellet was resuspended in 10 ml and then diluted with the same buffer to yield approximately 10 mg of protein/ml. Protein levels were determined by the Coomassie protein assay.

$^{36}Cl^-$ uptake was initiated by adding 200 μl of buffer containing $^{36}Cl^-$ (0.25 μCi) to a 200-μl aliquot of the membrane suspension which had been incubated for 15 min at 30°C. Four seconds after the addition of $^{36}Cl^-$, influx was halted by adding 4 ml of cold buffer and the solution was rapidly filtered through a Whatman GF/B filter under vacuum (20 in. Hg). An additional 8 ml of cold buffer was used to wash the filter. The filters were placed in 7 ml of Aquasol-2 (DuPont) scintillation fluid and the radioactivity was counted with a Packard Tricarb 460 CD liquid scintillation spectrometer. The average amount of $^{36}Cl^-$ bound to the filters in the absence of tissue was approximately 570 cpm. This value was defined as the filter $^{36}Cl^-$ retention and was subtracted from all sample results.

Statistical Methods

All cyclodiene levels, body weights, neurobehavioral, neurochemical, and testosterone data were analyzed using fixed effect factorial analyses of variance (general linear model) using the litter mean as the statistical unit. Normality of the data was assessed using the Kolmogorov–Smirnov test. Homogeneity of the variances was assumed. Body weights (days) and Cincinnati water maze (trials) data were analyzed using repeated measure ANOVA. Auditory startle reactions were analyzed using repeated measures on blocks. Cyclodiene levels in plasma, testosterone levels in serum, male mating behaviors, open field activity, and chloride-36 uptake were analyzed by nonrepeated measure ANOVA. Each endpoint was analyzed by sex (i.e., female and male data were analyzed separately). Comparisons between dosed groups and controls were tested using Fisher LSD. The comparison was considered statistically significant if the p value was less

TABLE 1
Concentrations of Analytes in Plasma at Various Developmental Periods (ng/ml)

Daily dose ng/g	Analyte	Dams postconception		Offspring postnatal		
		Day 20	Day 40	Day 41	Day 61	Day 81
Control	Heptachlor	ND[b]	ND	ND	4.3	7.2
	Heptachlor epoxide	0.1	ND	ND	ND	1.6
	trans-Chlordane	1.9	ND	ND	ND	1.5
	Oxychlordane	1.1	ND	ND	0.8	1.5
100	Heptachlor	1.7	0.8	F 1.3	ND	9.5
				ND	ND	4.2
	Heptachlor epoxide	1.3	ND	F 0.3	2.0	2.4
				M 0.2	0.9	2.3
	trans-Chlordane	8.5	1.9	F 4.7	9.3	13.4
				M 5.1	7.4	8.5
	Oxychlordane	6.6	0.9	F 5.3	4.8	8.0
				M 5.8	7.3	7.5
500	Heptachlor	0.6	ND	F 3.0	1.9	1.6
				M 3.0	ND	6.6
	Heptachlor epoxide	6.7	ND	F 5.9	4.3	8.9
				M 2.9	5.5	7.3
	trans-Chlordane	58.6	13.7	F 17.1[a]	42.0[a]	30.1[a]
				M 6.5	22.0	13.7
	Oxychlordane	54.5	16.6	F 31.9[a]	34.0[a]	34.6[a]
				M 15.0	25.7	24.5

[a] Significantly different from males ($p < 0.05$).
[b] ND, not detected.

than 0.05. All statistical analyses were performed using Number Cruncher Statistical System (Kaysville, Utah) or SAS-PC.

RESULTS

Sparging Procedure and Cyclodiene Levels in Serum and Milk

A sparging-gas chromatographic method was used to quantify the levels of heptachlor, heptachlor epoxide, *trans*-chlordane, and oxychlordane in plasma and milk samples. A summary of overall results is presented (Tables 1 and 2) and an illustration of the time course for heptachlor epoxide concentration in both the dams and offspring is shown (Fig. 1). In this exposure model, the plasma levels of heptachlor epoxide as well as the other cyclodienes increased during gestation and then decreased during lactation. In contrast, the plasma levels of the cyclodienes in offspring increased throughout the postweaning period.

The concentration of the cyclodienes is sex dependent. Female offspring at the 100 and the 500 ng/g dosed levels had greater concentrations of heptachlor epoxide, *trans*-chlordane, and oxychlordane than males, with significant differences for *trans*-chlordane and oxychlordane at the 500 ng/g dose level.

Active Testosterone

Testosterone levels were significantly reduced in the females at the 500 and 5000 ng/g exposure levels (Fig. 2). Testosterone levels were 20, 60, and 55% below control values for the 100, 500, and 5000 ng/g dosed groups, respectively. Dosed males showed very little effect with only a 10% decrease at the 5000 ng/g level.

Body Weights

Dose-related increases in body weight were observed in the female offspring dosed with 100, 500, and 5000 ng/g chlordane with increases in body weight of 8, 11, and 4%, respectively (Fig. 3). Only female offspring at the 500 ng/g level were significantly different from controls ($p < 0.05$)

TABLE 2
Concentration of Analytes in Dam Milk on Day 8 of Lactation (ng/ml)

Analyte	Daily Dose of Technical Chlordane	
	100 ng/g	500 ng/g
Heptachlor	ND[a]	33
Heptachlor epoxide	51	955
trans-Chlordane	2.4	333
Oxychlordane	46	1451

[a] ND, not detected.

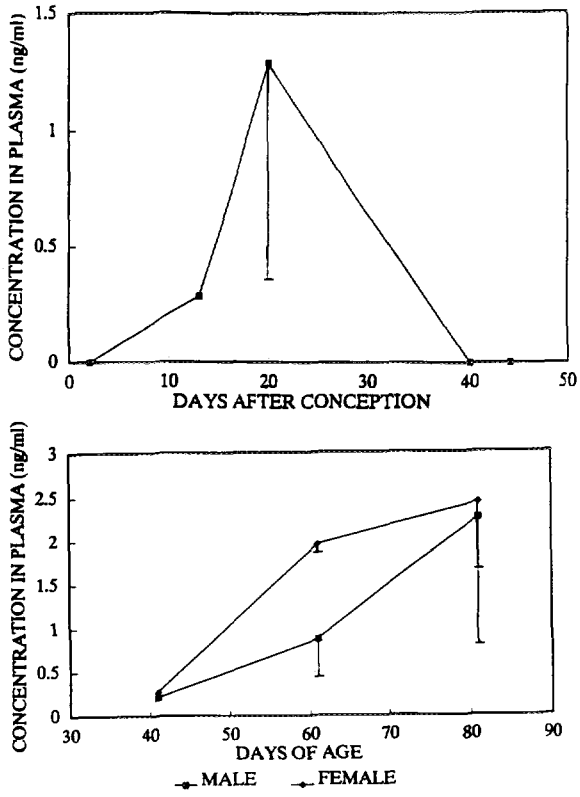

FIG. 1. Heptachlor epoxide concentration in the plasma of dams (top) and offspring (bottom) dosed daily with 100 ng/g technical chlordane (Mean ± SEM).

with five litters tested. Male offspring had only modest weight gains (Table 3). Nonsignificant weight gain was also observed in dams during gestation and lactation (Table 3). Chlordane's effect on body weight appears to be both dose and sex dependent.

Cincinnati Water Maze

All levels of chlordane-dosed female offspring committed significantly fewer errors than controls for all trials (i.e., 1–8) (Fig. 4). Trial 8 was deleted from the percentage of control in Figs. 4 and 5 due to the fact that all rats had learned the maze and small changes in the data resulted in large diagrammatic changes. Female offspring dosed at the 100 and 500 ng/g level completed the maze significantly faster than controls for all trials (Fig. 5). Also, chlordane-dosed females had significantly fewer failures in escaping the maze on trials 3 and 4 (Fig. 6). Chlordane-dosed male offspring were not significantly different from controls for any of the tests.

The error rates for all dosed females decreased noticeably beginning on the second test day with average error rates, expressed as percentage of controls, for the three replicates ranging from 78–96 on Day 1 (trial 1 and 2), to 53–73 on Day 2 (trial 3 and 4), and to 23–44 on Day 3 (trial 5 and 6) (Fig. 4). The same pattern was noted for time to escape in dosed females with averages decreasing from 94–100 on Day 1, to 65–89 on Day 2, and to 34–66 on Day 3 (Fig. 5). Also, chlordane-dosed females had a frequency of escape 40–80% higher than that of controls for trials 3 and 4 (Fig. 6). Only trials 3 and 4 were analyzed since rarely did an offspring escape on trials 1 and 2 and almost all escaped by trial 5. Although the differences were not significant, males also exhibited reduced errors and navigation times; however, the percentages were lower and more dose-dependent.

Mating Behaviors

Chlordane-treated males exhibited dose-dependent increases in male-typical mating behavior with the 500 ng/g group showing the greatest difference, 100 ng/g exhibiting slightly smaller differences, and 5000 returning closer to that of controls (Table 3). The 100 and 500 ng/g dosed males exhibited a significant reduction in latency to first intromission. Dose group 500 ng/g had a significant increase in both intromissions prior to ejaculation and total intromissions. Latency to intromission was reduced by 58% in the 100 ng/g, 64% in the 500 ng/g, and 13% in the 5000 ng/g dose groups. Intromissions prior to ejaculation increased 100% in the 100 ng/g, 150% in the 500 ng/g, and 100% in the 5000 ng/g dose groups. Total intromissions for each of these dose groups were even greater (Table 3).

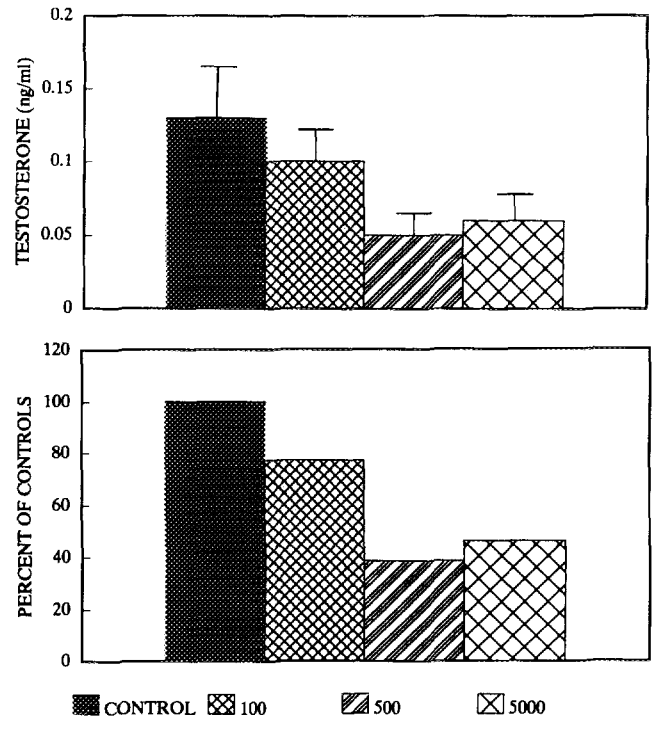

FIG. 2. Testosterone concentration in the plasma (top) and expressed as percentage of controls (bottom) of female offspring on PND 85. The 500 and 5000 ng/g dose groups were significantly different from controls, $p < 0.05$. Two-way ANOVA was conducted on means of litters, $n = 4$.

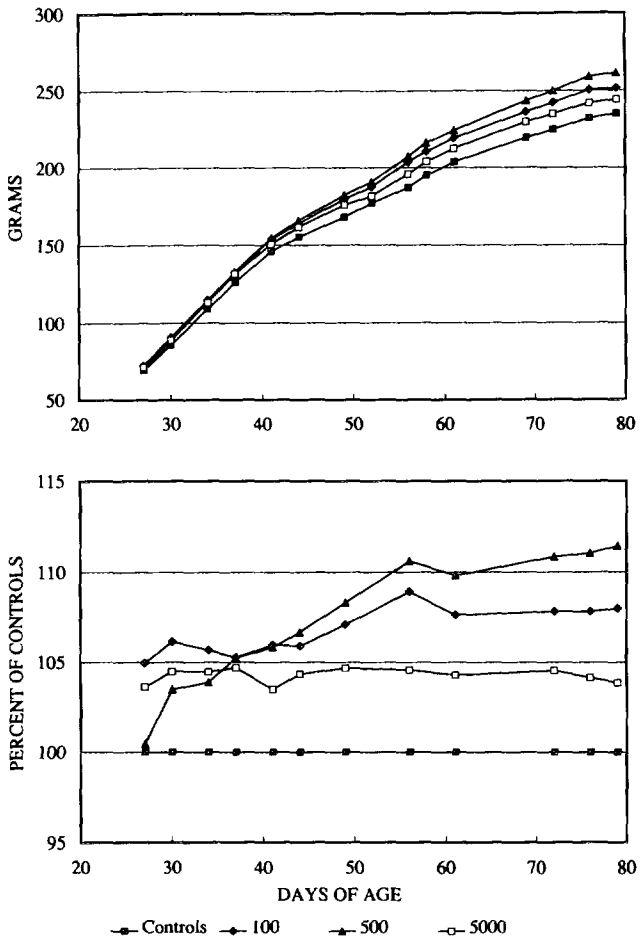

FIG. 3. Body weights expressed as actual weights (top) and percentage of controls (bottom) in female offspring. The 500 ng/g dose group was significantly different from controls, $p < 0.05$. Repeated measures two-way ANOVA (Days 27–79) was conducted on means of litters, $n = 5$.

Open Field Activity

Offspring activity exhibited a trend to vary with sex and dose. However, none of the dosed groups were significantly different from controls with five litters tested. For females, activity decreased 7% in the 100 and 500 ng/g dose groups, while a 5% increase in activity was observed in the 5000 ng/g group (Table 3). The activity of male offspring dosed with chlordane varied little from control values (Table 3).

Auditory Startle

Offspring were tested for auditory startle reactions on PND 81. Three parameters, average reaction V_{ave}, maximum reaction V_{max}, and latency to maximum reaction T_{max}, were evaluated for two separate test intervals—the first auditory evoked response (trial 1) and the next 50 auditory evoked responses (trials 2–51). The 50 evoked responses were further divided into 5 blocks of 10. Although the 100 and 500 ng/g dose offspring exhibited 20–60% increases in evoked responses for V_{ave} and V_{max} for both intervals, only the maximum response parameter for the 100 ng/g dosed group of block 1 was significantly different from that of controls (Table 3). Latency to maximum response in dosed offspring was not significantly different from that of controls (Table 3).

Chloride-36 Uptake

High-dose male rats had a significant reduction in uptake of chloride-36 into microsacs (Table 3). The 5000 ng/g dosed group had a 32% decrease in chloride uptake through $GABA_A$-mediated receptor complex channels as compared to that of controls. In pilot studies, positive and negative controls were used in *in vitro* studies following the same protocol except that ligands were incubated with the membrane microsacs for 30 min. The antagonists picrotoxin (10 μM) and heptachlor epoxide (10 μM) decreased chloride uptake by 46 and 48%, while the agonist GABA (100 μM) increased chloride-36 uptake by 29%. High-dose male offspring (5000 ng/g) had a substantial, although partial, reduction in chloride-36 uptake into microsacs.

TABLE 3
Test Results: Percentage of Deviation from Controls

		n, *F* Values	100 ng/g	500 ng/g	5000 ng/g
Body weights					
Gestation	Dams	5, 2.36	0	10 ↑	0
Lactation	Dams	5, 0.94	9 ↑	6 ↑	0
Offspring	Male	5, 0.22	0	2 ↑	3 ↑
Mating behavior					
Intromission latency	Male	3, 3.22	58[a] ↓	64[a] ↓	13 ↓
Intromissions prior to ejaculation	Male	3, 3.49	100 ↑	150[a] ↑	100 ↑
Total intromissions	Male	3, 2.84	200 ↑	280[a] ↑	180 ↑
Latency to ejaculation	Male	3, 0.89	10 ↓	15 ↓	0
Open field	Female	5, 1.38	7 ↓	7 ↓	5 ↑
	Male	5, 0.67	0	0	3 ↑
Auditory startle					
V_{ave}	Female	5, 1.87	43 ↑	78 ↑	43 ↑
(trial 1)	Male		18 ↑	42 ↑	47 ↑
T_{max}	Female	5, 0.42	9 ↓	4 ↓	1 ↓
(trials 2–51)	Male		0	7 ↑	4 ↑
V_{ave}	Female	5, 1.72	31 ↑	22 ↑	34 ↑
(trials 2–51)	Male		40 ↑	34 ↑	8 ↓
V_{max}	Female	5, 2.74	62[a] ↑	25 ↑	17 ↑
(trials 2–11)	Male		40[a] ↑	21 ↑	6 ↓
Chloride-36 uptake	Male	3, 15.6			38[a] ↓

[a] Significantly different from controls ($p < 0.05$).

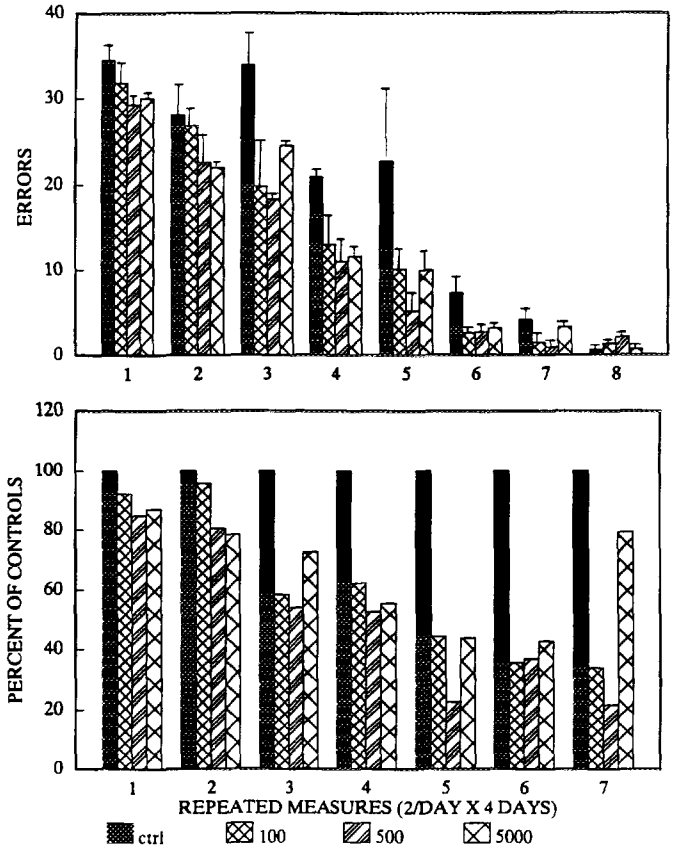

FIG. 4. Errors committed in the Cincinnati maze paradigm (PND 76–79) expressed as actual values (top) and percentage of controls (bottom) in female offspring. All dose groups were significantly different from controls, $p < 0.05$. Repeated measures two-way ANOVA (trials 1–8) was conducted on means of litters, $n = 3$.

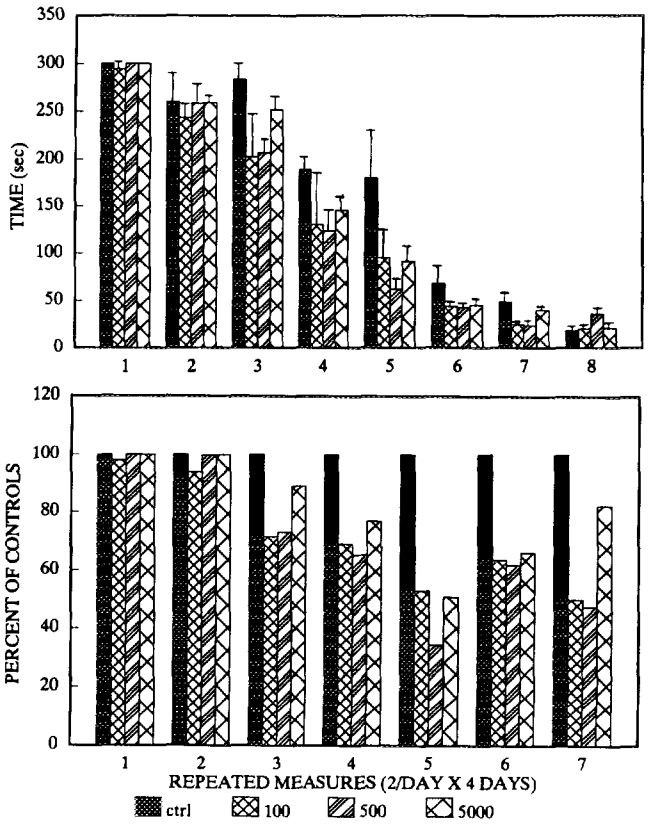

FIG. 5. Time to escape in the Cincinnati maze paradigm (PND 76–79) expressed as time (top) and percentage of controls (bottom) in female offspring. The 100 and 500 ng/g dose groups were significantly different from controls, $p < 0.05$. Repeated measures two-way ANOVA (trials 1–8) was conducted on means of litters, $n = 3$.

DISCUSSION

Many if not the majority of behaviors tested in rats are known to be sexually dimorphic (Beatty, 1979; van Haaren *et al.*, 1990). Of the tests conducted in this study, only auditory startle and chloride-36 uptake into brain microsacs have not been reported to vary with the sex of the rat. The results of this study correspond with previous reports on the male phenotype in that males metabolize and/or eliminate certain xenobiotics faster, weigh more, perform better on complex mazes (especially those paradigms that test reference memory), and exhibit male-typical mating behaviors (Beatty, 1979; van Haaren *et al.*, 1990; Waxman, 1988).

In this exposure model, the levels of cyclodienes in plasma of dams peaked on Day 20. After birth (Day 8), high levels of epoxides were secreted in the milk and apparently decreased serum levels during lactation. Sexually dimorphic organization of the rat brain is known to occur during Gestation Days 17–19 and during PND 1–10 corresponding to periods of high epoxide levels in dam plasma and milk (Baum *et al.*, 1988; Pang *et al.*, 1979). Although the developmental periods when sexually dimorphic organization in humans occurs are unknown, high levels of epoxide in fetal blood and mother's milk has been reported (Polishuk *et al.*, 1977; Savage *et al.*, 1981).

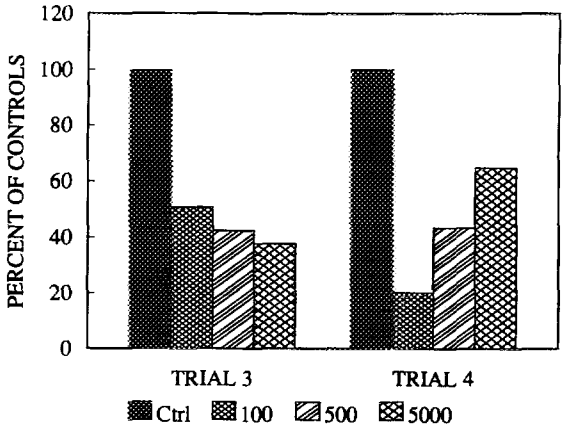

FIG. 6. Failure to escape in the Cincinnati maze paradigm (PND 77) expressed as percentage of controls in female offspring. All dose groups were significantly different from controls, $p < 0.05$. Repeated measures two-way ANOVA (trials 3 and 4) was conducted on means of litters, $n = 3$.

The concentration of cyclodienes in plasma was gender- and dose-related. Female offspring had higher concentrations of heptachlor epoxide, *trans*-chlordane, and oxychlordane than males. Adams *et al.* (1974) reported heptachlor epoxide levels in body fat of female rats three to five times the concentration in fat of males after feeding heptachlor epoxide for three generations. Likewise, Street and Blau (1972) reported that levels of oxychlordane in the adipose tissue of female rats were 15–20 times the levels found in adipose tissue of males fed diets containing *cis*- or *trans*-chlordane (50–200 ppm) for 11–15 days. These data suggest sexually dimorphic differences in hepatic microsomal enzymes that metabolize cyclodiene insecticides (Waxman, 1988).

Serum testosterone levels in offspring dosed with technical chlordane were gender- and dose-related. Earlier studies have demonstrated cyclodiene-induced increases in metabolism of sex steroids (Haake *et al.*, 1987), decreases in levels of sex steroids in various body compartments (Welch *et al.*, 1971), and decreases in levels of gonadotropins (FSH and LH) in sera of rats (Ateia *et al.*, 1990). These data suggest that chlordane induces microsomal P450 enzymes that hydroxylate steroids leading to increased elimination and reduces gonadotropins that stimulate testosterone synthesis. Diethylstilbestrol (DES), a potent estrogenic compound, has been reported to reduce testosterone levels in female rats on PND 56 when dosed during PND 1–5 (Halling and Forsberg, 1989). Taken together, these data suggest that chlordane may be affecting testosterone serum levels by mimicking estradiol or testosterone.

Chlordane-dosed offspring had gender- and dose-dependent increases in body weight. Similar results have been reported by Ambrose *et al.* (1953). In the Ambrose study, weanling rats fed seven concentrations of technical chlordane (10, 20, 40, 80, 160, 320, or 640 ppm) in food (w/w) for 420 days exhibited body weights that were gender- and dose-dependent. The low dose in Ambrose's study (10 ppm) equates to approximately the intermediate dose (500 ng/g) in this study. Females dosed at this level consistently weighed 10–20% more than the controls from puberty to the end of the study. Likewise, the 20 and 80 ppm-dosed female groups weighed 5–10% more than controls during postpubescence periods. In contrast, female rats dosed with 160 ppm or more showed a progressive decline in body weight. A different profile was reported for chlordane-dosed male rats with dose-related decreases in body weight. In another study, Witherup *et al.* (1955) fed rats heptachlor (1.5, 3.0, 5.0, 7.0, or 10.0 ppm) starting at 8 weeks of age and continuing for 2 years. In females, a dose-related increase in body weight was observed that was significantly different from that of controls. In contrast, males had a dose-related decrease in body weight that was significantly different from that of controls.

Weight gains in chlordane-treated offspring mirror results reported for neonate or adult female rats treated with estradiol, DES, testosterone, or the anabolic steroid, nandrolone phenylpropionate (NPP). Testosterone-dosed female rats at levels of 0.2 or 1 mg/kg/day had increased body weights, whereas testosterone doses greater than 10 mg/day showed no effect (Martinez *et al.*, 1984; Harvey and Hutchinson, 1973). Likewise, female rats dosed with 1, 4, or 10 mg/kg of NPP for 10 days had a 33% ($p < 0.01$), 17% ($p < 0.05$), or 12% increase in body weight (Choo *et al.*, 1991). A single injection of testosterone (10, 30, 90, or 270 μg) or estradiol shortly after birth increases body weight in females in an irreversible fashion (Bell and Zucker, 1971). This elevation in body weight is produced when neonates are dosed with steroids only on or before PND 3 (Tarttellin *et al.*, 1975). Female rats dosed on PND 2, 4, and 6 with DES had increased body weights, while males dosed in the same manner had decreased body weights (Lamartiniere *et al.*, 1988). Taken together, these data suggest that an isomer(s) of technical chlordane is(are) interacting with sex steroid receptors directly or altering the levels of sex steroids.

In the Cincinnati maze, chlordane-dosed offspring exhibited gender-related effects on all maze endpoints and dose-dependent effects on time to complete the maze. Dosed female offspring exhibited a consistent decrease in both error and failure to escape frequencies.

No studies could be found on the effects of chlordane or heptachlor on maze performance. However, Olsen *et al.* (1980), using a symmetrical maze described by Davenport *et al.* (1970), reported significant improvement in errors and total time in rat offspring dosed with dieldrin (0.35 ng/g/day) from Day 5 of gestation to PND 70 and tested on PND 70 and retested on PND 72. This suggests that even lower levels of technical chlordane would produce improved maze performance since 0.35 ng/g/day dieldrin is 1/300 the lowest dose of technical chlordane level used in the current study (100 ng/g/day).

The 100 and 500 ng/g dosed females exhibited similar numbers of errors and times on trials 1–8 as male controls. This suggests that at least one of the cyclodienes may be mimicking or increasing the level of estradiol. Estradiol has been established to be the proximal ligand that binds to estradiol receptors during perinatal periods and masculinizes hippocampus and cortex substrates that modulate spatial maze tasks (Williams and Meck, 1991). Estradiol is derived from testosterone intracellularly by the enzyme aromatase (McEwen *et al.*, 1977). Williams *et al.* (1989) demonstrated that if aromatase was inhibited with 1,4,6-androstatriene-3,17-dione (ATD), then males made more errors in the 12-arm radial maze. Several studies have shown that neonatal castration or treatment of male rats with cyproterone acetate (an inhibitor of testosterone) or neonatal androgenization of female rats within 10 days of birth resulted in maze learning efficiency in adulthood which resembled that of the opposite sex (Dawson *et al.*, 1975; Stewart *et al.*, 1975; Schenk and Slob, 1975).

The chlordane-dosed female offspring in the present study exhibited a similar decrease in number and pattern of errors as females treated with estradiol on PND 1, 3, 5, 7, and 9 (Williams and Meck, 1991). When compared to controls, chlordane-dosed female error rates decreased markedly starting on the second test day. However, this error rate varied little when offspring were retested an hour later. Similar decreases in errors committed by estradiol-treated offspring tested in a 12-arm radial maze were observed. These observations suggest that reference memory alterations account for a substantial portion of the overall effect and that chlordane alters levels of sex steroids or mimics sex steroids (Eckerman and Bushnell, 1992).

The sensitive period for the organizational effects of estradiol appears to be both pre- and postnatal. Williams *et al.* (1990) showed that female rats prenatally exposed to testosterone from male littermates (i.e., high percentage of males in litters) via placental circulation had maze performance no different from that of control males. Furthermore, the performance of females from low-male litters was indistinguishable from that of males castrated neonatally.

In tests for male-typical mating behavior in males, chlordane increased several behaviors (i.e., latency to first intromission, intromissions before first ejaculation, and total intromission) in a dose-dependent manner. Of the tests conducted, only mating behavior, visospatial, and auditory-evoked startle tests produced significant effects at the 100 ng/g levels. Interestingly, Gaulin and FitzGerald (1986) proposed an evolutionary hypothesis for sex differences in spatial ability which may link reproductive behavior and spatial cognition. They stated that sexual dimorphism in spatial ability was present in polygamous species (e.g., humans, rats, and meadow voles) in which females and males exploit different size ranges, whereas monogamous species (e.g., pine and prairie voles) lack sex differences in spatial ability and range patterns. Males with specialized spatial abilities may have been selected to defend a large territory needed for reproductive success. In contrast, females, which do not stray far from the nest, have a much smaller home range and presumably require a lower level of spatial ability. As in the radial maze test discussed earlier, rat neonates dosed with ATD on PND 1, 4, and 6 exhibited demasculinized male-typical mating behavior as adults. These males had significantly reduced ejaculation rates and lower preference scores for estrous females (Brand *et al.*, 1991).

Review of the literature did not reveal any studies on the effects of cyclodienes on mating behaviors. However, chlordane in mice dosed on PND 2, 3, and 4 has been shown to cause delayed vaginal opening, to increase ovarian weight, and to alter estrous cycles. Interestingly, the lower-dosed mice (75 μg) exhibited greater alterations than a higher dose (150 μg) (Talamantes and Jang, 1977).

An in-depth study of TCDD effects on male mating behavior, androgenic status, spermatogenesis, and reproductive capacity was recently published (Mably *et al.*, 1992a,b,c). Males exposed to TCDD perinatally had mating behaviors that were demasculinizing at 60, 75, and 115 days of age. In contrast to the present study, TCDD-dosed males had increased latency to intromission and increased latency to ejaculation at concentrations as low as 0.064 and 0.16 ng/g, respectively. TCDD-dosed males also had increased feminine behavior (i.e., increased lordosis, lordosis intensity, and luteinizing hormone plasma surge in response to progesterone injection) at concentrations as low as 0.40 ng/g. These effects were postulated to be caused by a reduction in the levels of plasma testosterone in male fetuses during the two critical periods of androgen-mediated differentiation (i.e., Gestational Days 17–21 and the first 2 hr after parturition) (Mably *et al.*, 1992a; Baum *et al.*, 1988; Pang *et al.*, 1979). The authors concluded that the male reproductive system of the rat is the most sensitive organ system studied thus far and that rats exposed perinatally produce adverse effects at 1/100 the TCDD level known to affect adult-exposed rats (Mably *et al.*, 1992a).

The concentration of oxychlordane and heptachlor epoxides in human fat is over 10,000 times the concentration of TCDD in human fat (Gross *et al.*, 1984; Kutz *et al.*, 1991). The levels of oxychlordane and heptachlor epoxide which produced altered mating behaviors in rats are found in the U.S. populace. This illustrates the necessity for further in-depth studies of these cyclodienes on (1) testosterone levels during critical periods, (2) testosterone and estrogen receptor numbers and responsiveness in neurons known to mediate copulatory behaviors, (3) androgen-mediated structures (e.g., anogenital distance; preoptic area of the hypothalamus), (4) mating behaviors of females, and (5) aggressive behavior in both males and females.

In the auditory startle test, chlordane-dosed offspring had increased response to an auditory signal. In another study, chlordecone, a cyclodiene derivative, has been evaluated for auditory startle reflex effects (PND 90) in rats dosed with 1 mg/pup on PND 4 (Mactutus and Tilson, 1985). No treatment effect was detected during initial auditory startle reflex assessment. However, sexually dimorphic effects of chlordecone on auditory startle reflex were revealed after stressing adult rats with harmine (a tremorogen). Chlordecone-exposed males were less responsive and chlordecone-exposed females were more responsive than same-sex vehicle-exposed littermates.

The chloride-36 uptake in dosed male offspring was significantly reduced. Previous investigators have reported that in *in vitro* studies chlordane, heptachlor, and their epoxides inhibit chloride-36 uptake into brain microsacs (Gant *et al.*, 1987; Bloomquist *et al.*, 1986; Abalis *et al.*, 1985). However, this is the first study to demonstrate decreased uptake of chloride-36 into brain microsacs prepared from intact rats previously dosed with chlordane. This result suggests that, at least for the 5000 ng/g dose, technical chlordane raises the resting membrane potential of neurons containing GABA-regulated chloride channels

and consequentially leads to neuronal hyperexcitability. Acute effects of chlordane are thought to occur by this mechanism. However, rats exposed to chlordane during the sexual organizational period(s) and also during postpubescent periods may exhibit dose-dependent effects due to competing mechanisms. For example, chlordane-induced masculinization would decrease activity, while adult rats exposed acutely would exhibit increased activity. Thus, increased activity may only appear at higher doses if chlordane-induced masculinization effects on activity predominate at lower doses.

The dimorphic phenotypes tested in this study are the result of sexual differentiation during a critical organization period in the rat brain and/or other tissues containing steroid receptors. The expression of many of these phenotypes can be modulated after pubescence (activational period) by steroids. In humans, the developmental periods for sexual differentiation of the CNS are not well understood. However, anatomically, the corpus callosum in human females is larger than that in males by 28 weeks of gestation (Jacobson, 1991). The critical periods for sexual differentiation in the rat are Gestational Days 17–19 and PND 1–10. During these periods, testosterone surges occur that bathe neurons and other tissues containing steroid receptors (Baum *et al.*, 1988; Pang *et al.*, 1979). Once in the cell, testosterone is converted to estradiol by the enzyme aromatase (McEwen *et al.*, 1977; MacLusky and Naftolin, 1981). Estradiol subsequently binds to nuclear receptors (Metzger *et al.*, 1991) and alters gene expression (Perheentupa and Huhtaniemi, 1990), neuron number, volume of brain structures, and synaptic connections (Gorski, 1986). Inhibition of the aromatization of testosterone to estradiol by an aromatization inhibitor (1,4,6-androstatriene-3,17-dione) or blocking estradiol receptors during the critical periods of organization results in incomplete masculinization of behavior (McEwen *et al.*, 1977). From these studies, it is now known that sexually dimorphic structures and functions arise from estradiol interaction with estradiol receptors during critical developmental periods. A few reports have suggested that dihydrotestosterone, the 5-α reduced metabolite of testosterone, augments this sexual differentiation.

Estradiol and androgen receptors are expressed in a variety of brain regions (e.g., medial preoptic area, ventral hypothalamus, amygdala, lateral septum, hippocampus, and cortex) which form complexes with steroids. These complexes interact with genomic material altering gene expression and ultimately determining sexually dimorphic phenotypes (Kaplan and McGinnis, 1989). Unlike other areas of the CNS where these receptors are known to be present during perinatal and into adult periods, the hippocampus does not express these receptors after PND 10 (O'Keefe and Handa, 1990). This lack of estradiol receptor in the hippocampus during the activational period may explain the lack of dose-dependent effects seen in the Cincinnati maze compared to other tests.

Several lines of evidence suggest that cyclodienes are mimicking sex steroids and/or altering their levels. (1) Only chlordane-dosed females were significantly different from controls in cyclodiene levels in serum, in the Cincinnati water maze, and in body weight. Dosed females exhibited more masculine effects which many times mirrored dose-dependent effects observed for steroid-treated rats. (2) Chlordane-dosed males exhibited increased male-typical mating behaviors. (3) The 5000 ng/g dosed males had decreased chloride-36 uptake. Previous results reported by several investigators showed that the cyclodienes bind to putative steroid receptors on the $GABA_A$ complex and alter $^{36}Cl^-$ uptake into neurosynaptosomes.

The 100 and 500 ng/g chlordane-dosed groups exhibited dose-related changes in body weight and Cincinnati maze, auditory startle, and mating behavior, while the 5000 ng/g dosed group deviated less from control values. From these results it appears that competing mechanisms are functioning in which the overall effect is the sum of more than one factor. Several possible mechanisms could contribute to the observed dose-dependent effects. (1) Cyclodienes are known to inhibit GABAergic inhibition. (2) Cyclodienes could be mimicking sex steroids and consequently affecting many steroid-mediated processes during developmental periods (organizational) and/or during later periods (activational). (3) Concomitantly, cyclodienes could be altering the level of these endogenous steroids by altering synthesis and elimination processes. (4) Additionally, cyclodienes could change the number and responsiveness of endogenous steroid receptors. Earlier studies have shown that chlordane and other cyclodienes alter sex–steroid-mediated processes such as levels of microsomal enzymes in the liver, sex organ development and function, and gonadotrophin levels. It is consistent with these previous findings that these cyclodienes would bind with sex–steroid receptors in CNS and alter various sexually dimorphic behaviors and functions.

The current lowest observable adverse effect level (LOAEL) for less serious effects in animals dosed orally with chlordane between 15 and 364 days is approximately 1000 ng/g for neurological effects and 5000 ng/g for developmental effects (U.S. Department of Health and Human Services, 1993). This study has demonstrated changes in a variety of behaviors and functions which are 10–50 times lower than the current LOAEL. Even a lower LOAEL might have been observed if lower doses were tested using the USEPA recommended guideline of 20 litters per dose.

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